THE HEMATOPOIETIC STEM CELL NICHE IN LEUKEMIA PREDISPOSITION

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About the book design:
Artistic impression of the bone marrow, in which the main discoveries of this dissertation were made. The bone marrow (red edges) is a complex organ accommodating hematopoietic stem cells and their niches, surrounded by a shell of bone (white cover), penetrated by blood vessels (red texts) and nerves (yellow pages).

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To my parents

献给我的父母

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1

GENERAL INTRODUCTION

1.1 HEMATOPOIESIS

Hematopoiesis entails the generation of blood. Blood is a liquid organ composed of cells with non-dispensable roles in vertebrates: red blood cells (RBCs) transport oxygen, white blood cells (WBCs) fight against infections, and platelets (PLTs) facilitate wound healing. All the functional mature blood cells are generated from hematopoietic stem and progenitor cells (HSPCs). By definition, hematopoietic stem cells (HSCs) can self-renew and differentiate to give rise to all blood lineages. HSCs do so by gradual loss of lineage potential and transit into progenitor cells, which further differentiate into mature blood cells. In this subchapter, the evolution of our perspectives on hematopoiesis will be reviewed; furthermore, the development and function of neutrophils, which is an important cell type involved in immunity and inflammation, will be discussed in detail.

1.1.1 Hematopoietic stem and progenitor cells

The classical model of hematopoiesis has been organized into a tree like structure, in which HSCs are considered as a homogeneous pool, residing atop of the lineage tree, and forming a highly hierarchical structure with downstream progenitor cells. Recent advances in technology have challenged the classical view of hematopoiesis and provided novel insights, including early lineage branching points and heterogeneity in HSCs. Therefore, it is important to rethink hematopoiesis from different perspectives.

The evolving picture of hematopoiesis

The classical tree model of hematopoiesis was shaped around the year 2000, in which lymphoid and myeloid progenitor populations downstream of HSCs were characterized.[1,2] In this model, long-term HSC (LT-HSC) was defined by its ability to repopulate blood for a prolonged period. LT-HSC transits into short-term HSC (ST-HSC) upon receiving signals required for differentiation, which subsequently lose their multilineage potential and give rise to progenitor cells in a stepwise manner. The first branching point of the lineage tree was proposed to segregate lymphoid lineages (common lymphoid progenitors, CLPs) from myeloid lineages (common myeloid progenitors, CMPs).[1,2] Subsequently, CLPs branch further to form unipotent progenitor cells which give rise to the mature cells of the lymphoid lineage, including T cells, B cells, and NK cells.[1] In contrast, CMPs branch further to form oligopotent progenitors, including general myeloid progenitors (GMPs), which give rise to granulocytes, monocytes, and dendritic cells; and megakaryocyte-erythroid progenitors (MEPs) which give rise to megakaryocytes and erythrocytes.[2] The classical tree model of hematopoiesis is still used in many textbooks nowadays **(Figure 1A)**.

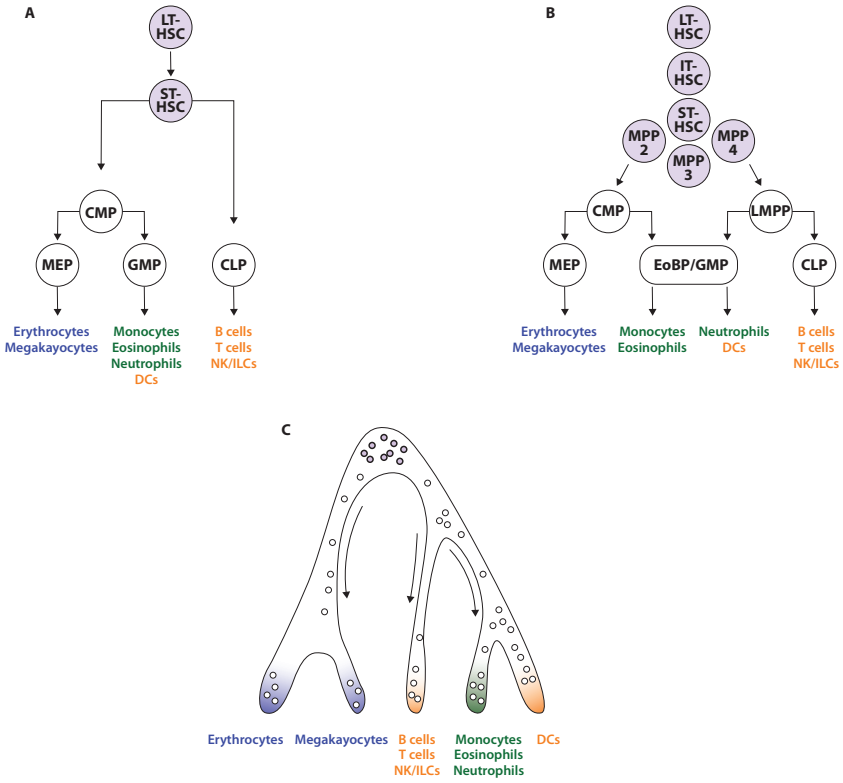

Figure 1. The evolutionary view of hematopoiesis. Adapted from Laurenti and Göttgens.[16] A. The classic tree model of hematopoiesis shaped around the year 2000. B. The modified tree model of hematopoiesis shaped around the year 2010. C. The continuum model of hematopoiesis shaped after the year 2015.

In the following decade, the subsequent introduction of novel immuno-phenotypical markers **(Table 1)** and other experimental approaches suggested several modifications to the classical tree model **(Figure 1B)**. LT-HSC, intermediate-term HSC (IT-HSC) and ST-HSC were distinguished depending on their long, immediate, and short-term self-renewal capacity in transplantation studies.[3-6] HSCs restrict their self-renewal capacity and give rise to multipotent progenitors (MPPs).[3,6] MPPs have been subdivided into a few distinct populations, including MPP2, MPP3, and MPP4, based on different lineage biases.[7,8] Alternative nomenclatures of MPPs have been proposed, as hematopoietic progenitor cells (HPCs).[9] Moreover, the segregation of myeloid and lymphoid fate has become more fluent, suggested by the identification of intermediate lymphoid-primed multipotent progenitors (LMPPs).[10,11] However, there has been evidence arguing against the incorporated classical tree model. For example, a study of individual HSC clones has proposed the existence of HSCs intrinsic lineage biases, which is preserved during self-renewal.[12] Also, the early branching of megakaryocytic lineage has been demonstrated.[13,14] Furthermore, the analysis of lineage output frequencies has suggested much higher percentage of progenitors are unipotent.[15]

The advances in RNA-sequencing and single-cell technologies have contributed to our understanding and revealed new insights into the model of hematopoiesis.[17-20] Data from these single cell studies suggest that the HSC pool is heterogeneous at both molecular and functional levels. Therefore, a continuum model of hematopoiesis is emerging, which proposes the HSC compartment consist a heterogeneous pool of cells organized hierarchically. HSCs do not jump from one developmental stage to another, instead, they gradually lose plasticity and acquire lineage biases in a continuous manner, meanwhile maintaining the flexibility to adapt dynamic changes in the need of blood **(Figure 1C)**.[17,19] The continuum model is compatible with the classical tree model and later modifications, also in line with previous conclusions including early lineage segregation, transcriptional lineage programming, and functional lineage bias. Important discoveries shaping the molecular and cellular mechanisms of HSCs and progenitors will be addressed more in details in the following paragraphs.

Table 1. HSCs and MPPs defined by immune-phenotypical markers in mouse and human

Species	Name	Cell-surface phenotype	Self-renewal	Differentiation	References
Mouse	LT-HSC	Lin-Sca1+ckit+CD34-CD150+CD135-CD48- ±EPCR+ ±Rholo	High		3, 9
	ST-HSC/ MPP1/MPP	Lin-Sca1+ckit+CD150-CD48- ±CD135-	Low		4, 7, 8
	MPP2/HPC2	Lin-Sca1+ckit+CD150+CD48+ ±CD135-	Low	Lymphoid deficient	7-9
	MPP3/HPC1	Lin-Sca1+ckit+CD150-CD48+ ±CD135-	Low	Balanced	7-9
	MPP4	Lin-Sca1+ckit+CD135+CD150-CD48+	Low	Lymphoid biased	7, 8
Human	LT-HSC	Lin-CD34+CD38-CD45RA-CD90+CD49f+ ±Rholo	High		6
	ST-HSC/MPP	Lin-CD34+CD38-CD45RA-CD90-CD49f-	Low	Balanced	6

Adapted from Laurenti and Göttgens.[16] LT, long-term; ST, short-term.

HSC and progenitor boundaries

HSCs are fundamentally characterized by two essential properties: self-renewal and multi-lineage differentiation capacity. Self-renewal entails the capacity to generate HSCs themselves via divisions, whereas differentiation entails the capacity to produce mature cells of all blood lineages. Operationally, the function of HSCs is determined by their long-term multi-lineage reconstitution ability in bone marrow transplantation experiments performed in irradiated recipient mice. The threshold for duration is defined arbitrarily, but a minimum of 16 weeks after transplantation is generally accepted in the field; more stringently, LT-HSCs

retain the potential to engraft and repopulate all the blood lineages at least in secondary recipients following transplantation.[3] In contrast, progenitors lack the capacity of extended self-renewal; they have restricted lineage differentiation potential, and are lost within 2-3 weeks after primary transplantation.[6]

The distinct properties of HSCs and progenitors could be attributed to both cell intrinsic and extrinsic mechanisms. Intrinsically, distinct transcriptional landscapes have implicated the differences between HSCs and progenitor cells: abundant evidence has demonstrated that lineage bias is intrinsically stable and is maintained following transplantation experiments.[21,22] The extrinsic mechanisms are mainly mediated by the bone marrow microenvironment, also known as the niche. HSCs and progenitors occupy distinct regions in the bone marrow, which are different in cellular composition and spatial organization.[23] As a consequence, HSCs and progenitors might be exposed to different extrinsic signals, such as cytokine gradient and cell-cell contact, which could lead to distinct self-renewal and lineage commitment. In line with this, several reports have demonstrated distinct niches for HSCs and HPCs: HSCs reside in a quiescent niche, whereas HPCs reside in a more active niche.[24,25]

Heterogeneity in HSCs

HSCs were previously thought to be a homogenous population; however, later findings have demonstrated that substantial heterogeneity exists in the pool of HSCs. For example, a series of single-cell transplantation studies have illustrated that the majority of HSCs have a pre-determined fate towards certain lineages.[14,26,27] Recent advances in RNA-sequencing have provided opportunities to investigate the transcriptome in single HSC. Single cell profiling studies have indicated that distinct transcriptome programs exist among HSCs and hematopoietic lineages in metabolic and cell cycle status.[18,19]

The causes of HSC heterogeneity can be attributed to different mechanisms. Cellular stress has been implied in shaping the molecular and functional heterogeneity among HSCs by regulating self-renewal and lineage bias.[28,29] LT-HSCs remain quiescent under homeostasis and are associated with anaerobic metabolism.[30,31] A metabolically inactive state and glycolysis reduces the level of reactive oxygen species (ROS) and protects HSCs against DNA damage.[32] Moreover, cytokines are known to be involved in supporting the survival and maintenance of HSCs.[33] Recent evidence has suggested cytokines, including macrophages colony stimulating factors (M-CSF/*CSF1*) and granulocyte colony stimulating factor (G-CSF/*CSF3*), can instruct lineage fate in HSCs.[34,35] In line with this, myeloid and lymphoid biased HSCs respond differently to cytokines.[36,37] Last but not least, biophysical properties can add another layer of complexity in HSCs heterogeneity. For example, it has been shown that the composition and stiffness of extracellular matrix (ECM) regulate HSCs by altering the accessibility of cytokines and growth factors.[38,39]

HSCs during development and ageing

At different developmental stages HSCs exhibit distinct properties. During embryonic development of the mouse, HSCs are first generated in the aorta-gonad-mesonephros (AGM) region, subsequently they migrate to the fetal liver at E14.5, and finally arrive in the bone marrow at birth.[40] A major difference between embryonic and adult HSCs is the proliferative rate. HSCs in the fetal liver are highly proliferative, whereas in the adult they are largely quiescent.[40] Therefore, it is conceivable that functionality difference might exist between fetal and adult HSCs, although all the adult HSCs are likely to be the direct descendants of fetal HSCs.

Hematopoiesis alters dramatically in ageing as reflected by myeloid skewing, lymphoid deficiency, and expansion of immune-phenotypical HSCs in aged animals. These ageing-related phenotypes are likely to be driven by the clonal expansion of myeloid-biased HSCs.[41,42] Moreover, a single-cell transcriptome profiling and transplantation study has revealed the molecular lineage priming and functional lineage bias towards platelets in aged HSCs.[43] Mechanistically, the accumulation of DNA damage, shortening of telomeres, and alteration in metabolic states have been reported to contribute to the ageing related phenotypes of HSCs.[44,45]

Aside from the HSC intrinsic programs, the HSC niche could also contribute to the alterations in hematopoiesis during development and ageing. Under distinct developmental stages, the sites where HSCs reside are substantially different in their cellular components and vasculature properties, thus potentially contributing to the divergent regulation of HSCs.[40,46] Furthermore, the mesenchymal organization in bone marrow is drastically altered during ageing, as evidenced by the decreased frequencies of cellular components involved in supporting HSCs.[23] Thus, the combination of HSC intrinsic and extrinsic programs contribute to the ageing-related alterations in hematopoiesis, resulting in impaired immunity, and increased propensity to develop hematological malignancies.

1.1.2 Neutrophils

Neutrophils are essential cellular components for the immunity against microbes in vertebrates; they are the most abundant type of circulating leukocyte, comprise 10-25% and 50-70% of mice and human peripheral blood, respectively.[47] To maintain sufficient quantities in the blood, large numbers of neutrophils are produced, estimated to be 2×10^{11} cells per day in adult humans.[48] Neutrophils are generated in the bone marrow from myeloid progenitors and subsequently released in the circulation as post-mitotic cells. Once micro-organisms have succeeded in penetrating the skin and mucosa membrane to enter the host, neutrophils will be recruited and activated by endothelial cells at the site of infection to perform their microcidal and immune regulatory functions. Neutrophils have a short lifespan and are readily cleared in the host at distinct sites after exerting their functions.

Neutrophil development

The production of monocytes and granulocytes is termed myelopoiesis, which is a major part of hematopoiesis, to maintain the large turnover. Myelopoiesis takes place in the bone marrow, where HSPCs reside. The production of neutrophils from committed myeloid progenitors is termed granulopoiesis, which includes a series of stages: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and polymorphonuclear (segmented) cell **(Figure 2)**.[48]

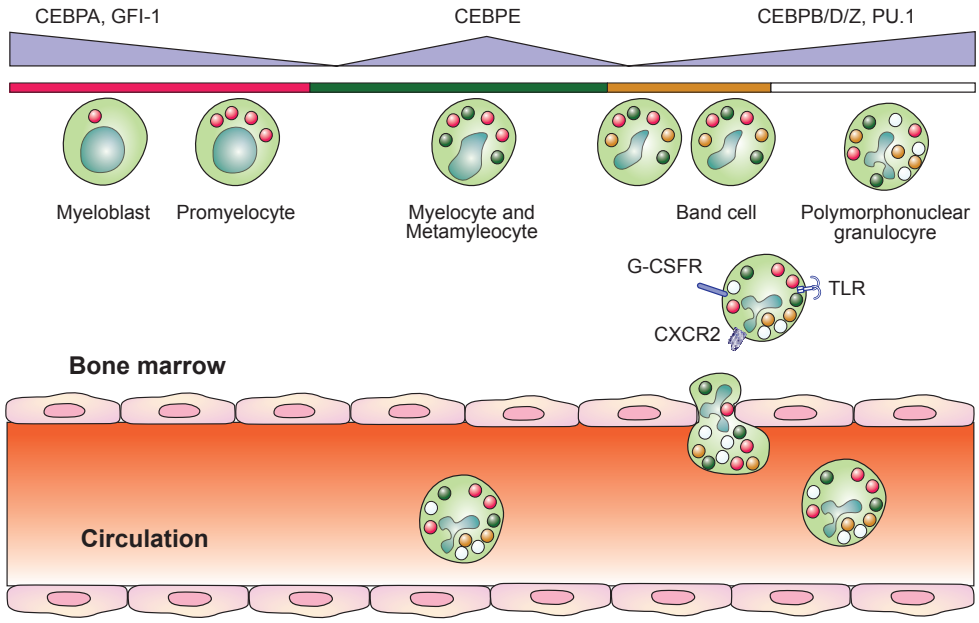

Figure 2. The development and release of neutrophils. Adapted from Borregaard.[48] Committed myeloid progenitors differentiate to mature neutrophils via the following stages: myeloblast, promyelocyte, myelocyte, metamyelocyte, band cell, and polymorphonuclear cell. The expression levels of transcription factors required for granulopoiesis, including PU.1, GFI-1 and CEBP family of transcription factors fluctuate through different developmental stages, as represented by the gradient triangles on top of the figure. Primary granules (red), secondary granules (green), tertiary granules (yellow), and secretory vesicles (white) are produced at distinct stages of granulopoiesis, as illustrated by the colored bars and spheres. Mature neutrophils are released into circulation under the regulation of CXCR2, TLR, and G-CSFR.

Several transcriptional factors are essential for granulopoiesis, mainly PU.1 and CCAAT/enhancer binding protein (CEBP) family of transcription factors. PU.1 is indispensable for myeloid lineage commitment; disruption of *PU.1* in HSCs caused the absence of early myeloid progenitors, whereas its disruption in CMPs/GMPs caused a blockage in myelomonocytic differentiation.[49,50] The expression of PU.1 is observed throughout granulopoiesis and gradually increases towards maturation.[51] The balance of PU.1 and CEBPA determines lineage decision towards granulocytes.[52,53] CEBPA is absolutely required for granulopoiesis, whose expression peaks at the GMP stage, gradually decreasing and diminishing upon

terminal differentiation.[54,55] CEBPA regulates the expression of various molecules, including microRNAs, transcription factors, and growth factors.[56,57] Growth factor independent-1 (GFI-1) is another important transcription factor necessary for neutrophil differentiation.[58,59] GFI-1 has been demonstrated to suppress progenitor transcriptional programs by repressing genes encoding HOXA9, MEIS1, and PBX1.[60] Meanwhile, GFI-1 induces granulopoiesis by repressing transcription factors implicated in macrophage differentiation, including EGR-1 and EGR-2.[61]

CEBPE is essential for terminal granulopoiesis and maturation of neutrophils.[62,63] The transition from promyelocyte to myelocyte is associated with cell cycle exit.[64] CEBPE regulates this process by binding to Rb protein and E2F1 to repress their transcriptional activity in driving the cell cycle.[65] Moreover, CEBPE is essential for the production of secondary granule proteins at the myelocyte stage;[66] mutations in CEBPE leads to deficiency of specific granules.[67] Other CEBP family of transcription factors also play important roles in granulopoiesis, including CEBPB, CEBPG, CEBPD, and CEBPZ, whose expression is observed from the metamyelocyte stage onward and peak in mature cells.[51] The CEBP family of transcription factors are regulated by dimerization and phosphorylation, thus enabling the individualized content of granule proteins during granulopoiesis.[48]

Another important aspect of neutrophil maturation is the sequential formation of granules, starting from the promyelocyte stage. Three types of neutrophil granules are formed consecutively, including primary (azurophilic) granules which contain myeloperoxidase (MPO), secondary (specific) granules which contain lactoferrin, and tertiary (gelatinase) granules which contains matrix metalloproteinase-9 (MMP-9).[68] Finally, at the band cell and polymorphonuclear cell stage, secretory vesicles containing membrane proteins are produced, which play pivotal roles in the neutrophil-mediated inflammatory response.[69]

It is noteworthy that under both homeostatic and emergency situations such as acute infection, granulopoiesis is regulated by cytokines, including granulocyte-macrophage colony-stimulating factor (GM-CSF/*CSF2*), G-CSF/*CSF3*, interleukin-3 (IL-3) and IL-6, acting on committed myeloid progenitors.[70] Together with the stress-induced cell cycle activation of HSCs,[71,72] emergency granulopoiesis provides a boost to hematopoiesis in fighting the excessive amount of invading pathogens.

Neutrophil function

Upon maturation, neutrophils are released into the circulation to perform their functions. Two cytokine receptors expressed on myeloid cells, C-X-C motif chemokine receptor 4 (CXCR4) and CXCR2 are important in this process. The ligand of CXCR4 is CXCL12.[73] CXCR4 is required for maintaining mature neutrophils in the bone marrow; deletion of *Cxcr4* resulted

in increased number of neutrophils in the circulation.[74] The ligands for CXCR2 are CXCL1 and CXCL2, which are produced by endothelial cells.[75] Deletion of *Cxcr2* resulted in retention of neutrophils in the bone marrow.[75] The release of mature neutrophils is regulated by CXCR2, G-CSF receptor (G-CSFR) and Toll-like receptors (TLRs) during the final stages of neutrophil maturation.[76] G-CSF has been shown to downregulate CXCL12 while upregulate CXCL1 and CXCL2 in bone marrow endothelial cells, therefore tilting the balance towards neutrophil release.[75,77]

Mature neutrophils released in circulation are post-mitotic. Upon the entry of pathogens into the host, signals generated by microbes and tissue-resident macrophages will activate endothelial cells at the side of infection, which will in turn recruit neutrophils and activate them to perform their antimicrobial function. Neutrophils can eliminate pathogens by intracellular and extracellular mechanisms. For intracellular killing, neutrophil can eliminate microorganisms by phagocytosis; when pathogens are encapsulated in phagosomes, neutrophils can use ROS or release antibacterial proteins (cathepsin, defensin, and lysosome) to kill the pathogens.[48] Highly activated neutrophils can generate neutrophil extracellular traps (NETs) to eliminate extracellular microorganisms. NETs are composed of a core DNA element attached by histone and enzymes (lactoferrin, cathepsin, MPO and NE) released from neutrophil granules.[78] NETs can kill pathogens directly with antimicrobial proteins, or immobilize pathogens and facilitate subsequent phagocytosis of trapped microorganism by recruiting neutrophils and macrophages.[79,80]

Neutrophils are short-lived cells, with an average lifespan up to 12.5 hours in mice under homeostatic conditions.[81] However, the lifespan of activated neutrophils is considerably increased to ensure their presence during inflammation.[82] Aged neutrophils have increased expression of CXCR4, which may facilitate their migration into the bone marrow and elimination by macrophages.[83] Finally, the unique developmental, circadian and migration properties suggest an emerging role for neutrophils in regulating the HSC niche,[84] together with many other cell types, which will be addressed in the next subchapter.

1.2 THE HEMATOPOIETIC STEM CELL NICHE

In stem cell biology, the niche is defined as the local tissue microenvironment which maintains and regulates stem cells. The HSC niche plays important roles in communicating the needs for hematopoiesis to the HSCs. The HSCs niche varies during development due to the distinct sites of hematopoiesis. During embryonic development, the HSC niche migrates through the AGM region, yolk sac, placenta, fetal liver and spleen, to be finally localized in the bone marrow.[40] In adulthood, the HSC niche is located in the bone marrow under homeostasis; upon hematological stress, extramedullary niches have been documented in the spleen and liver.[23,85] In this subchapter, the main cellular components of the HSC niche and their identification will be introduced, to provide the scientific background and rationale to the research questions proposed in this dissertation.

1.2.1 Cellular components of the HSC niche

The recent discovery of specific surface markers, advancement in microscopy and genetic mouse modeling, as well as the development of molecular techniques have enabled in-depth understanding of the HSC niche. A large variety of cellular components have been identified in the bone marrow and are proposed to maintain distinct HSC populations **(Figure 3)**.

Mesenchymal stem/stromal cells

Mesenchymal stem/stromal cells (MSCs) are major components of the HSC niche **(Table 2)**. MSCs are capable of differentiation and give rise to osteolineage cells, chondrocytes, adipocytes, and myocytes.[87] Moreover, MSCs have been characterized by the capacity to form fibroblast colonies (CFU-F) in semi-liquid cultures.[88] Given that most HSCs colocalize with blood vessels in the bone marrow,[3,89] it is reasonable to hypothesize that stromal cells surrounding blood vessels promote the maintenance of HSCs. The first perivascular cells identified to regulate HSCs were CXCL12-abundant reticular (CAR) cells, which surround sinusoids and localize close to HSCs.[90] The ablation of CAR cells led to reduced number of HSCs, accompanied by impaired adipocytic and osteolineage differentiation potential of the bone marrow.[91] Pericytes surrounding arterioles have also been identified as a HSC niche component; these arteriolar pericytes express NG2 and have been proposed to be important for the maintenance of HSC quiescence.[92,93]

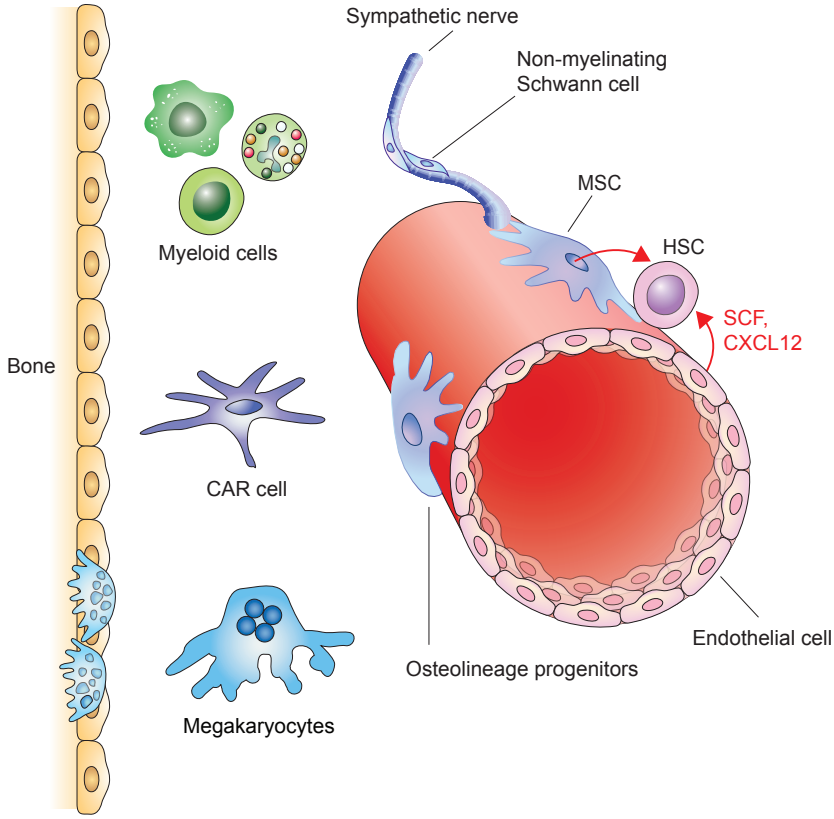

Figure 3. The hematopoietic stem cell niche. Adapted from Morrison and Scadden.[86] The HSC niche is predominantly located in the bone marrow through adulthood. Several cellular components of the HSC niche are illustrated, including MSCs (CAR cell, osteolineage progenitor) endothelial cell, certain myeloid cells (macrophage, Hdc$^+$ myeloid cell, and neutrophil), megakaryocyte, nerve cell, and non-myelinating Schwann cell. HSCs are proposed to be maintained by direct cell-cell contact and secreted factors.

Further evidence supporting the view of MSCs as part of the HSC niche has been provided by the identification of Nestin-expressing cells in the bone marrow.[94-96] Cells expressing the *Nestin*-GFP transgene have the ability to form CFU-F and can be propagated as non-adherent mesenspheres.[94] Moreover, these *Nestin*-GFP$^+$ cells localize close to HSCs and highly express HSC maintenance genes; the ablation of *Nestin*-GFP$^+$ cells resulted in reduced HSC numbers.[94] Similarly, another mesenchymal cell identified as a HSC niche component was the Leptin receptor (LepR) expressing cell; LepR$^+$ cells express low levels of *Nestin*-GFP but high levels of HSC maintenance genes including *Scf* and *Cxcl12*.[97] Moreover, an additional *Nestin*-negative multipotent mesenchymal progenitor was identified by expression of paired related homeobox 1 (Prx1), a transcription factor implied in early limb bud mesenchyme development.[25,98] Although *Nestin*-GFP$^+$, LepR$^+$, and Prx1$^+$ cells were identified independently, they might have functional overlaps which remain to be completely defined.

The contribution of primitive mesenchymal cells in regulating hematopoiesis has been further elucidated by the conditional deletion of important niche factors for HSCs in distinct mesenchymal populations. Deletion of *Scf* in *Nestin*$^+$ mesenchymal progenitors (targeted by *Nestin*-Cre or *Nestin*-CreER) did not affect the frequency and function of HSCs, whereas the deletion of *Scf* in *Nestin*-negative mesenchymal progenitors (targeted by *Lepr*-Cre) resulted in depletion of HSCs.[99] Along similar lines, deletion of *Cxcl12* from *Nestin*-negative mesenchymal progenitors (targeted by *Lepr*-Cre or *Prx1*-Cre) resulted in a significant loss of HSCs, as well as a significant decrease of common lymphoid progenitors.[24,25] These studies suggest that distinct mesenchymal progenitors, as well as distinct perivascular stromal cells, are important for the maintenance and regulation of HSCs.

Osterix-expressing mesenchymal progenitors comprise another subset of mesenchymal cells in the bone marrow. Osterix, encoded by the *Sp7* gene, is a transcription factor required for osteoblast differentiation and bone formation.[100] Lineage tracing studies have demonstrated the contribution of *Osterix*$^+$ cells to the developing and adult bone marrow. Embryonic *Osterix*$^+$ cells contribute to nascent bone tissues, as well as transient stromal cells with mesenchymal multipotency.[101,102] Perinatal *Osterix*$^+$ cells give rise to osteolineages and long-lived bone marrow stroma; these stromal cells have functional overlap with *Nestin*-GFP$^+$ MSCs, including CFU-F forming and tri-lineage differentiation *in vitro*.[102] Adult *Osterix*$^+$ cells are osteolineage restricted and do not contribute to other stromal populations.[102,103] Osteolineage progenitors are associated with vascularization of the developing bone, implying their potential interaction with HSCs.[104] In line with this, perinatal deletion of *Osterix* resulted in the elimination of hematopoietic cells in the trabecular-rich metaphysis, suggesting the presence of osteolineage progenitors is important for hematopoiesis.[105] Similarly, depletion of *Osterix*$^+$ cells during embryonic development resulted in hyper-proliferative HSCs and failure to engraft in transplanted recipients.[106] However, the function of HSCs was normal when *Cxcl12* was deleted in *Osterix*-expressing cells (targeted by *Osterix*-cre), suggesting that factors other than CXCL12 are mediating the effects of *Osterix*-expressing cells (and their downstream progeny) on HSCs.[25]

Endothelial cells

The bone marrow is a highly vascularized organ. The existence of endothelial niches was first proposed by Kiel *et al.* based on the close proximity of HSCs and endothelium.[3] Approximately 85% of HSCs are located in the proximity of sinusoids, and most HSCs are distant from arterioles and transition zone vessels.[89] *In vitro* studies have suggested a role for endothelial cells in the maintenance and proliferation of HSCs.[107-109] *In vivo* support for endothelial contributions to HSC behavior was provided by Yao *et al.*, showing that targeted deletion of cytokine receptor subunit gp130 from endothelial cells resulted in bone marrow hypocellularity, extramedullary hematopoiesis and reduced HSC numbers.[110] Moreover,

the regeneration of sinusoidal endothelial cells is important for the engraftment of HSPCs following transplantation.[111] Importantly, Ding *et al.* and Greenbaum *et al.* have provided further evidence that endothelial cells regulate HSCs, demonstrating that targeted deletion of *Scf* or *Cxcl12* in endothelial cells (targeted by *Tie2*-Cre) depleted HSCs from the bone marrow.[24,25,99] These studies all suggest that endothelial cells regulate HSCs and comprise an important component of the HSC niche.

Table 2. Bone marrow MSCs associated with the HSC niche

Cell population	Included cell types	Location	Time when present	References
CAR cells	Nearly all cells that express high levels of CXCL12 and SCF; and LepR+ cells	Perivascular, mainly perisinusoidal but also periarteriolar	Characterized in adult bone marrow	90
NG2+ stromal cells	Stromal cells, osteoblasts, Schwann cell, osteocytes and chondrocytes	Periarteriolar, as well as associated with nerve fibres and the endosteum in adults	Fetal liver and adult bone marrow	92, 93
Nestin-GFP+ stromal cells	Nestin-GFPlow cells include LepR+/CAR cells; Nestin-GFPhi cells include stromal cells, Schwann cells and endothelial cells	Nestin-GFPlow perisinusoidal cells and Nestin-GFPhi periarteriolar cells	Characterized in fetal and adult bone marrow	92, 94-96
LepR+ stromal cells	Nearly all cells that express high levels of CXCL12 and SCF	Perivascular, mainly perisinusoidal but also periarteriolar	Beginning in postnatal bone marrow and persisting in adult bone marrow	97, 99
Prx1+ stromal cells	LepR+/CAR cells, osteoblasts	Perivascular and endosteal	Arise during fetal development and persist in adult bone marrow	24, 25
Osterix+ osteolineage progenitors	Mesenchymal progenitors	Perichondrium and perivascular	Characterized in fetal and postnatal bone marrow	101, 102

Adapted from Crane *et al.*[23] CAR cell, CXCL12-abundant reticular cell; CXCL12, CXC-chemokine ligand 12; SCF, stem cell factor; NG2, neural–glial antigen 2; GFP, green fluorescent protein; LepR, leptin receptor; Prx1, paired-related homeobox 1.

Myeloid cells

Several types of myeloid cells have been implied in regulating HSCs. Macrophages are important in retaining HSCs in the bone marrow by regulating CXCL12 production; depletion of macrophages in the bone marrow resulted in the mobilization of HSCs.[112,113] Subsets of macrophages have been implied in the preservation of quiescent LT-HSCs via regulating ROS levels or transforming growth factor β 1 (TGF-β1)/Smad3 signaling.[114,115] Importantly, a recent study has demonstrated that committed myeloid cells expressing histidine decarboxylase (Hdc^+) play important roles in maintaining myeloid-biased HSCs, via a histamine-dependent

feedback loop.[116] Last but not least, aged neutrophils have been shown to infiltrate the bone marrow; depletion of neutrophils led to increased number of CAR cells, suggesting a role for neutrophils as regulators of the HSC niche.[83]

Megakaryocytes

Megakaryocytes (Mks) have been demonstrated to regulate HSC function via multiple mechanisms. Mks localize adjacent to HSCs non-randomly in mouse bone marrow.[117] Ablation of Mks activated quiescent HSCs and increased HSC proliferation, proposing a role of Mks in inhibiting HSC cell division.[117,118] Mechanistically, Mks negatively regulate HSC proliferation via CXCL4 production; *Cxcl4*$^{-/-}$ mice demonstrated a similar HSC phenotype as Mks depleted mice, whereas the administration of recombinant CXCL4 resulted in reduced HSCs proliferation associated with increased quiescence.[117] Alternatively, the mechanism of HSC activation upon Mks depletion could also be explained by reduced TGFβ signaling.[118] Moreover, Mks have been demonstrated to expand and upregulate fibroblast growth factor (FGF) production upon stress to promote the proliferation and maintenance of HSCs.[119] This data suggests that Mks are a hematopoietic cell derived component of the HSC niche.

Neurons and glial cells

Nerve fibers and non-myelinating Schwann cells can regulate HSCs via indirect mechanisms. Nerve fibers have been implicated in promoting the survival of HSC niche components and initiating hematopoietic recovery after chemotherapy.[120] Furthermore, nerve fibers control the daily cyclical release of HSCs in the bloodstream by reducing *Cxcl12* expression in the HSC niche.[121] Non-myelinating Schwann cells are glial cells of the peripheral nervous system; they are in contact with HSCs, express genes encoding niche factors, and maintain HSC dormancy by regulating latent TGF-β signaling.[122]

1.2.2 Surface markers identifying mesenchymal niche cells in the bone marrow

Technical advances in flow cytometry have prompted the discovery of specific immuno-phenotypical markers in the non-hematopoietic compartment of the bone marrow, which contributed to the identification and purification of multiple cell types in the HSC niche. Thomson *et al.* demonstrated that bone marrow cells labeled by CD271 (low-affinity nerve growth factor receptor) can give rise to all three germ layers.[123] Subsequently, Cattoretti *et al.* demonstrated the labeling of stromal cells in fetal and adult bone marrow by CD271.[124] A decade later, Jones *et al.* demonstrated that CD271 labeled cells in the human bone marrow are enriched for CFU-F activity.[125,126] Another important surface molecule identified in the human bone marrow is CD146 (melanoma-associated cell adhesion molecule), which labels adventitial reticular cells capable of forming CFU-F *in vitro* and promoting hematopoietic support in xenograft transplantation models, suggesting the identification of a robust MSC population.[127] Subsequently, Tormin *et al.* demonstrated both CD271 single

positive or CD271/CD146 double positive cells can give rise to stromal cells *in vitro* and form hematopoietic stroma *in vivo*, suggesting CD271 is a *bona fide* marker for identifying human MSCs.[128]

In parallel studies, the combination of CD90 (THY-1), CD106 (vascular cell adhesion molecule 1) and CD271 has been shown to enrich for clonogenic cells; clonal characterization of CD90/CD271 double positive cells revealed functional heterogeneity within these clones.[129] Moreover, two independent studies have reported that clonogenic stromal cells in the human bone marrow which are capable of forming mesenspheres can be identified by either CD105 (Endoglin)/CD146 or CD51 (Integrin alpha V)/CD140α (platelet-derived growth factor receptor alpha).[130,131] These mesenspheres can robustly differentiate into mesenchymal lineages *in vitro*, and can enhance the expansion and function of primitive hematopoietic cells when co-cultured under undifferentiated conditions. However, it is important to note that the CD51/CD140α double positive cells have been identified in fetal but not adult bone marrow in humans.[131]

The non-hematopoietic cells in mouse bone marrow are typically enriched as $CD45^-$, $Ter119^-$ and $CD31^-$ triple negative cells (TNCs).[94,103,132,133] CD45 is a type I transmembrane molecule expressed on all nucleated hematopoietic cells,[134] except erythroid progenitors, which can be labeled by Ter-119, an antigen associated with glycophorin A.[135] Subsequently, endothelial cells can be excluded using CD31, also known as platelet endothelial cell adhesion molecule (PECAM-1).[136] TNCs cells represent approximately 0.5% of adult mouse bone marrow cells and are thought to be predominantly composed of stromal cells and mesenchymal progenitors.[102,131]

The subsequent identification of positive selection markers for bone marrow stromal cells has improved the isolation of specific niche populations with higher purity. Morikawa *et al.* demonstrated that the combination of Sca-1 and CD140α labels the non-hematopoietic compartment in adult mouse bone marrow with enriched CFU-F capacity, whereas Sca-1 and CD140α double negative cells have restricted potential to differentiate into osteogenic cells.[132] Moreover, CD51 in combination with CD140α was found to isolate MSCs.[131] In parallel studies, the expression of CD105 has been demonstrated to be the minimal requirement to establish a hematopoietic niche, suggesting mesenchymal progenitors are labeled with CD105.[137,138]

1.2.3 Factors mediating the crosstalk between niche and HSC

Investigation of niche factors required for the maintenance of HSCs, such as CXCL12, stem cell factor (SCF), and thrombopoietin (TPO), has provided important insights in the localization and function of putative HSC niche components.

CXCL12 is a chemo-attractant formerly known as stromal cell-derived factor 1 (SDF1). The interaction between CXCL12 and its receptor CXCR4 is required for the initiation of hematopoiesis in the bone marrow.[139,140] Global deletion of *Cxcl12* or *Cxcr4* in adult mice reduced quiescent HSCs.[90,141,142] CXCL12 expression has been confirmed in the HSC niche, such as MSCs and endothelial cells, although with substantially different expression levels.[25,143-145]

SCF is the ligand for tyrosine-protein kinase KIT.[146-148] SCF exists in soluble and membrane-bound forms, both are required to maintain HSCs locally.[149-151] Moreover, SCF is also required for *ex vivo* culture of HSPCs.[152] Therefore, analyzing the expression pattern of SCF could provide insights in identifying putative HSC niche components. In line with this, data from *Scf*-GFP mice revealed that SCF is predominantly expressed by MSCs surrounding sinusoids and endothelial cells in the bone marrow.[99]

TPO is the ligand for myeloproliferative leukemia protein (MPL). TPO-MPL signaling is required for HSC maintenance,[153-156] as well as megakaryocyte and platelet development.[144,157,158] However, the expression of TPO is limited in adult bone marrow under homeostasis but much higher in liver and kidney.[157,159] Therefore, it remains elusive whether HSCs are maintained by TPO produced locally or from a distance site.

Many other factors which regulate HSC non-cell-autonomously have been identified in the bone marrow, such as angiogenin (ANG),[160] dickkopf-1 (DKK1),[161] and Notch 2.[162] These factors are dispensable for HSC maintenance under homeostasis but are implicated in regeneration after injury. Isolation and characterization of niche cells located in the vicinity to engrafted HSCs could facilitate the discovery of novel niche factors involved in regeneration. Moreover, long-range factors such as hormones synthesized in distant organs have also been implicated in modulating HSCs or niche function.[163]

Taken together, multiple cellular components of the HSC niche have been identified, some of which can be purified by specific immune-phenotypical markers. HSCs are maintained by the niche via direct cell-cell contact and/or secreted factors. The HSC-niche interaction is also implicated in the development and progression of bone marrow disorders, which will be discussed in the following subchapters.

1.3 BONE MARROW FAILURE SYNDROMES AND LEUKEMIA PREDISPOSITION

The hematopoietic system is maintained through carefully orchestrating HSC intrinsic and extrinsic programs during the lifespan of a host. Tilting of the delicate balance could result in the development of hematological diseases. In this subchapter, a selection of hematological disorders will be introduced. Moreover, the genetics and molecular pathogenesis of these disorders will be discussed, to provide the scientific background and relevance for the research questions proposed in this dissertation.

1.3.1 Bone marrow failure syndromes

Bone marrow failure (BMF) syndromes are hematological disorders characterized by insufficient production of functional blood cells in specific blood lineages. BMF syndromes can be inherited or acquired after birth. Congenital BMF syndromes are rare, caused by germline mutations involved in fundamental cellular processes; whereas acquired BMF syndromes are more common, associated with somatic mutations in hematopoietic cells.

Shwachman-Diamond syndrome

Congenital defects in genes encoding proteins associated with ribosome biogenesis have been related to BMF syndromes, including Shwachman-Diamond syndrome (SDS), Dyskeratosis Congenita (DC), and Diamond-Blackfan anemia (DBA), collectively known as ribosomopathies. Ribosomes are ribonucleoprotein complexes which catalyze protein synthesis by translating mRNAs into encoded protein products. The eukaryotic ribosome comprises the 40S small subunit and the 60S large subunit, which are composed of structural ribosomal RNAs (rRNAs). Ribosome biogenesis is a highly complex and biosynthetic energy consuming process in which the 40S small subunit and 60S large subunit join together to form the active 80S ribosome, in cooperation with small nucleolar RNAs (snoRNAs), ribosomal proteins, and assembly factors.[164]

SDS is an autosomal-recessive disorder characterized by BMF, exocrine pancreatic insufficiency, and skeletal abnormalities.[165-168] Neutropenia is a hallmark of the hematological abnormalities in SDS patients, although anemia and thrombocytopenia have been reported.[169,170] The bone marrow of SDS patient is typically dysplastic and hypocellular.[171] The incidence of SDS has been estimated to be 1 in 77,000 individuals.[172] Strikingly, SDS patients have a high propensity to develop myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML), with a median age of 18 years, and a 30% cumulative risk at the age of 30 years.[173,174] SDS patients are monitored closely for leukemia progression. G-CSF and blood transfusion are common treatments to manage cytopenia. To treat exocrine pancreatic insufficiency SDS patients are given pancreatic enzyme supplements. Bone marrow transplantation is reserved for patients with severe BMF or MDS/AML progression.[164]

The causative gene for SDS is the Shwachman-Bodian Diamond syndrome (*SBDS*) gene. Approximately 90% of SDS patients carry mutations in the *SBDS* gene.[175] Constitutive homozygous or compound heterozygous 183-184 TA>CT and 258+2 T>C are the most prevalent mutations found in SDS patients, causing premature termination of *SBDS* transcription.[176] These mutations result in decreased levels of *SBDS* transcripts associated with reduced amount of functional SBDS proteins. SBDS protein is involved in the hydrolysis of GTPase elongation factor-like 1 (EFL1) and catalyzing the removal of eukaryotic initiation factor 6 (eIF6), which is essential for the maturation of 60S large subunit **(Figure 4)**.[177,178] In line with this, the mutations in *SBDS* results in a lower amount of actively translating 80S ribosomes and a reduction in global translation rate.[179]

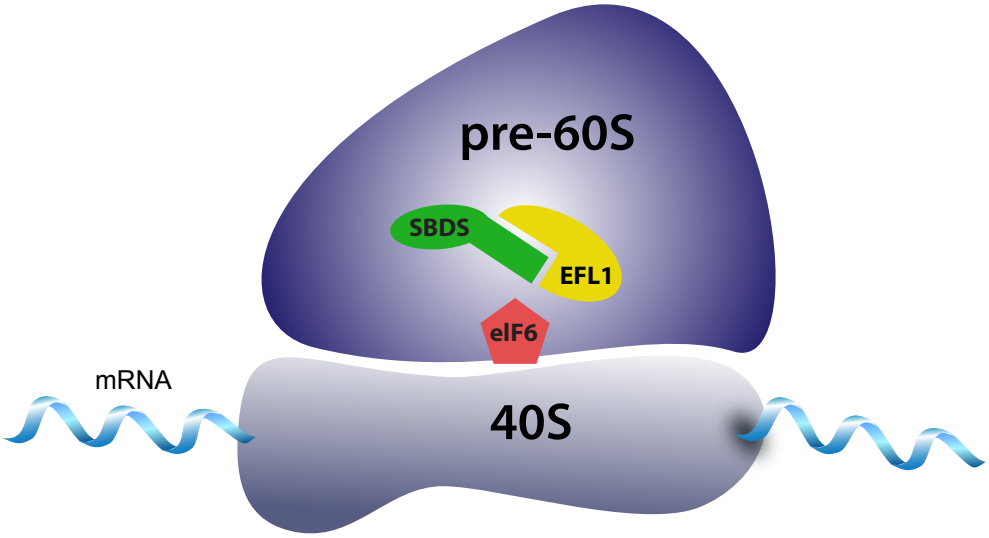

Figure 4. Defects in SBDS essential for ribosome biogenesis is associated with SDS. Adapted from Finch *et al.*[178] The active eukaryotic 80S ribosome is composed of the 40S small subunit and the 60S big subunit. The ribosome biogenesis requires the removal of eIF6 from the pre-60S subunit mediated by SBDS and EFL1. Mutations in the SBDS gene leads to defects in ribosomal biogenesis associated with SDS.

It remains an open question how defects in *SBDS*, a factor involved in ribosomal biogenesis in all cells, result in physiological perturbations in specific organ systems, including the hematopoietic system. Tremendous effort has been made to address the molecular mechanisms underlying SDS pathogenesis. RNA interference (RNAi) mediated *Sbds* inhibition in primary hematopoietic cells led to impairment of granulopoiesis *in vitro* and reduction of myeloid progenitors *in vivo.*[180] Short-term hematopoietic engraftment was impaired partially due to reduced homing of defective HSPCs to the bone marrow.[180] In another study, it has been demonstrated that the inhibition of *SBDS* in HSPCs resulted in a reduction of erythroid differentiation partially due to increased ROS levels and insufficiency in translation.[179]

Moreover, human pluripotent stem cell models of SDS faithfully recapitulate the deficiencies in hematopoietic differentiation as well as the exocrine pancreatic development *in vitro*, suggesting protease-mediated autodigestion as a mechanistic link between the defects in these two body systems.[181] The first mammalian model of neutropenia in SDS was reported via transplantation of HSPCs carrying targeted deletion of *Sbds* in *Cebpa* expressing cells, pinpointing the differentiation block of granulopoiesis at myelocyte stage, mechanistically associated with the activation of tumor suppressor p53 and induced apoptosis in myelocytes.[182] The mechanism under which SDS patients are predisposed to leukemia progression remains poorly understood. A recent report has demonstrated that clonal hematopoiesis due to somatic mutations in *TP53* was present in 48% SDS patients compared to 0% healthy controls, suggesting that the acquisition of *TP53* mutations in SDS is likely an initiating event in the progression to MDS/AML.[183] MDS and leukemic transformation, however, have not been observed in hematopoietic cell autonomous models of SDS, suggesting that ancillary cells may play an essential role in this.

Myelodysplastic syndromes

MDS are the most prevalent form of acquired BMF syndromes, characterized by ineffective hematopoiesis of one or more lineages in the bone marrow, and are associated with increased risk for transformation to AML. The incidence of MDS has been estimated to be 2-12 in 100,000 individuals and higher in elderly people.[184] Ineffective hematopoiesis is associated with anemia, infection, and bleeding.[185] The bone marrow of MDS patients is typically hypercellular, with dysplasia in hematopoietic cells, and <20% of blasts.[186] Increased apoptosis in the MDS bone marrow has been reported.[187] According to the International Prognostic Scoring System (IPSS), MDS patients can be scored based on the extent of cytopenia, percentage of blasts in the bone marrow, and cytogenetics; subsequently, patients can be categorized into different risk groups defined by clinical outcomes, including median survival and time to AML evolution.[188,189] Treatments for low-risk MDS (LR-MDS) typically consists of transfusions, growth factors and, in specific cases, lenalidomide.[190,191] The treatment for high-risk MDS (HR-MDS) is typically hypomethylating agents such as azacitidine (AZA),[192] or if possible, intensive chemotherapy followed by allogeneic stem-cell transplantation.[193,194] Approximately one-third of MDS patients progress to AML.[186]

The exact cause of MDS remains largely unknown. Increased risk has been reported in patients with historical exposure to hazardous chemicals and radioactive therapy.[195] High-throughput genomic sequencing and analysis have characterized a number of genes recurrently mutated in MDS patients, including genes encoding splicing factor (*SF3B1*), epigenetic regulators (*TET2*, *ASXL1*, *DNMT3A*), and transcription factors (*RUNX1*, *TP53*).[196-200]

1.3.2 Acute myeloid leukemia

AML is a malignant disorder of the hematopoietic system characterized by clonal expansion and abnormally differentiated blasts of myeloid lineage in the blood and bone marrow. The accumulation of immature myeloid cells leads to impairment of normal hematopoiesis, resulting in anemia, severe infection, and hemorrhage in AML patients.[201] The incidence of AML has been estimated to be 4 in 100,000 individuals.[202] Clinically, AML is diagnosed by >20% of blasts in the bone marrow.[203] In addition, AML can be further categorized based on karyotype and molecular aberrations. Due to the heterogeneous nature of this disease, genomic and molecular aberrations are assessed in AML patients to guide the appropriate therapy.[204] Classically, a combination of cytarabine and anthracycline has been the standard regimen for intensive chemotherapy.[205,206] However, emerging new drugs for treating AML provide alternatives.[207] Moreover, aggressive supportive management is required for other relevant complications in AML, such as tumor lysis syndrome and intravascular coagulation.[208]

AML can arise *de novo*, or evolve from patients with a history of other hematological disorders such as BMF syndromes.[207] Moreover, AML may arise in patients with a previous history of cytotoxic chemotherapy or irradiation.[209] The molecular mechanisms underlying leukemogenesis remain incompletely understood; it is generally accepted that AML originates from HSPCs endowed with the capacity of self-renewal. The acquisition of specific driver mutations in HSCs is likely to occur early in leukemic transformation, providing a selective advantage for the clonal expansion of leukemic stem cells (LSCs) and progression to AML.[210] Several recurrent mutations in AML have been identified in genes encoding nucleocytoplasmic shuttling protein (*NPM1*), epigenetic regulators (*TET2*, *ASXL1*, *DNMT3A*, *EZH2*, *IDH1*, *IDH2*), transcription factors (*RUNX1*, *CEBPA*, *TP53*), splicing factor (SFS3R), tyrosine kinase receptor (*FLT3*), and cohesion complex (*STAG2*, *RAD21*).[201] Somatic mutations in specific epigenetic regulators including *TET2*, *ASXL1*, and *DNMT3A* have been identified in preleukemic HSCs decades before AML progression, suggesting they are the early events driving leukemogenesis.[211-213] These somatic mutations have also been identified in expanded clones in the peripheral blood of elderly individuals without hematological malignancy, introducing a phenomenon termed clonal hematopoiesis of indeterminate potential (CHIP); individuals carrying CHIP mutations have increased risk for developing hematological malignancies.[214-216]

Taken together, both congenital and acquired BMF syndromes manifest impairments in hematopoiesis, and in some cases, predisposition to AML. Monogenic congenital BMF syndromes could provide valuable models to investigate the molecular mechanisms underlying the pathogenesis of MDS and leukemic evolution.

1.4 THE HSC NICHE IN BMF SYNDROMES AND LEUKEMIA PREDISPOSITION

Many longstanding observations have challenged the view that (pre)leukemic conditions are entirely driven by genetic events in hematopoietic cells as 'stand-alone' mechanism. In MDS, for example, xenotransplant models using immunodeficient mice have consistently shown poor engraftment of myelodysplastic cells and failure to confer BMF or leukemic phenotypes. Similarly, cell autonomous mouse models of SDS have not recapitulated myelodysplasia or leukemic evolution. These observations have sparked a long-standing debate about a potentially causative or permissive role of the microenvironment in leukemogenesis.[217] More recently, the pathogenesis of BMF syndromes and their leukemic progression have been associated with the interaction between HSCs and their niche.[218] Two conceptual mechanisms through which the HSC niche contributes to hematological malignancies, niche induced oncogenesis and niche facilitated oncogenesis, will be introduced in this subchapter.

1.4.1 Niche induced oncogenesis

Niche induced oncogenesis entails the scenario in which the primary event occurs in the non-hematopoietic cells; subsequently, this mutagenic niche transforms healthy or pre-disposed hematopoietic cells into leukemic cells.

Experimental support for this model comes from models in which genetically engineered mutations in the HSC niche components induce hematopoietic neoplasm in mice. Raaijmakers *et al.* have provided the first proof of principle for niche induced oncogenesis by targeted deletion of *Dicer1* in osteolineage progenitors, which disrupted the integrity of hematopoiesis in mice, led to myelodysplasia and in some cases progression to AML.[219] In line with this, Kode *et al.* demonstrated the activation of β-catenin signaling in osteoblasts induced alterations in the differentiation of hematopoietic progenitor cells, and led to the development of AML with non-random chromosomal aberrations.[220] Similarly, Dong *et al.* demonstrated the introduction of *Ptpn11* activating mutation in mesenchymal and osteolineage progenitors, but not in mature osteoblasts and endothelial cells, induced a transplantable myeloproliferative neoplasm (MPN).[221] A commonality of these studies is that only the genetic disruption, specifically of immature mesenchymal (bone progenitor) cells in the HSC niche resulted in disease phenotypes, suggesting a key role for these cells in preserving the integrity of the hematopoietic tissue. Collectively, these studies have provided experimental evidence that primary alterations of the HSC niche can result in secondary neoplastic disease.

1.4.2 Niche facilitated oncogenesis

Niche facilitated oncogenesis defines a scenario in which the primary event of leukemogenesis occurs in the hematopoietic cells; subsequently, the mutated hematopoietic cells transform

the healthy niche into a mutagenic niche to further promote the clonal expansion of leukemic cells.

This concept is supported by experimental evidence from genetic mouse and xenograft transplantation models. For example, LSCs have been shown to reduce CXCL12 expression in the bone marrow niche, which was associated with a growth advantage to the leukemic cells.[222,223] Moreover, Schepers *et al.* demonstrated that LSCs from MPN remodeled the endosteal niche into a leukemic niche, favoring LSC expansion and promoting bone marrow fibrosis while impairing normal hematopoiesis.[224] Similarly, pathogenic alterations of the sympathetic nervous system triggered by LSCs in the bone marrow have been implicated in creating a mutagenic niche favorable for the expansion of leukemic cells.[223,225] Medyouf *et al.* demonstrated that MSCs derived from MDS patients manifested disturbed differentiation programs; these MDS-derived MSCs are essential for the propagation of MDS-initiating HSPCs, and healthy MSCs adopted MDS features when exposed to MDS-derived hematopoietic cells.[226] Mechanistically, a recent study has demonstrated that MSCs from low-risk MDS patients exhibited enriched transcriptional signatures in cellular stress, and upregulated expression of genes encoding inflammatory factors with inhibitory effects on hematopoiesis.[227] Thus, emerging experimental evidence suggests that primary alterations in hematopoietic cells may trigger the activation of inflammatory programs in the HSC niche, subsequently driving the pathogenesis and leukemic progression in BMF syndromes.

1.5 AIM OF THIS DISSERTATION

As described in this chapter, our view of hematopoiesis has evolved drastically in the last decade. The classical model of hematopoiesis has been updated with early lineage separation and pronounced heterogeneity in the HSC pool. A comprehensive set of cellular components in the HSC niche have been identified and demonstrated to fulfill essential roles in maintaining normal hematopoiesis. Contributions of the HSC niche to normal and malignant hematopoiesis have become evident. Hematopoietic cell extrinsic factors have been implied in the pathogenesis of BMF syndromes and hematological malignancies. This dissertation aims to investigate the molecular mechanisms underlying hematopoietic cell extrinsic (HSC niche) contributions to BMF and leukemogenesis, and elucidate their relevance to human disease.

In **chapter 2**, we provide mechanistic insights into niche-induced oncogenesis in a mouse model of SDS. Mice with targeted deletion of *Sbds* in *Osterix*-expressing mesenchymal progenitors recapitulated key features of SDS patients in the marrow and bone. Inflammatory alterations in niche cells are shown to drive these characteristics, including genotoxic stress in HSPCs. Broader relevance of this concept of niche induced genotoxic stress in heterotypic stem cells is demonstrated in LR-MDS patients, where niche inflammation identified patients at risk for leukemic transformation.

The upstream drivers of inflammatory programs in the mesenchymal compartment of LR-MDS patients remain largely unknown. In **chapter 3**, we identify NF-κB signaling as a major driver of the mesenchymal inflammation phenotype in LR-MDS. Transcriptional profiling of LR-MDS mesenchyme has revealed the activation of NF-κB signaling; the functional consequences of this on HSPCs are interrogated in an *ex vivo* co-culture system with genetically modified mesenchymal cells.

In **chapter 4**, we address the contribution of cytopenia to BMF syndromes and leukemogenesis. Neutropenia is a hallmark of SDS and other BMF disorders with the propensity to transform to AML. In this chapter, we test the hypothesis that neutropenia in itself causes replicative and genotoxic stress in HSCs, thus contributing to their exhaustion and malignant transformation. In the SDS mouse model of profound and sustained neutropenia, we, unexpectedly, did not find experimental support for this hypothesis. Rather, HSC function is conserved and even augmented in neutropenia, shedding further light on the mechanisms of 'emergency' hematopoiesis and identifying myeloid cells as a putative niche component with negative effects on HSC maintenance.

Finally, in **chapter 5**, the main findings of this dissertation are summarized and put into context with contemporary knowledge in the field, accompanied by future perspectives to point out directions for follow-up studies.

REFERENCES

1. Kondo, M., Weissman, I.L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91**, 661-672 (1997).
2. Akashi, K., Traver, D., Miyamoto, T. & Weissman, I.L. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* **404**, 193-197 (2000).
3. Kiel, M.J*., et al.* SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**, 1109-1121 (2005).
4. Kiel, M.J., Yilmaz, O.H. & Morrison, S.J. CD150- cells are transiently reconstituting multipotent progenitors with little or no stem cell activity. *Blood* **111**, 4413-4414; author reply 4414-4415 (2008).
5. Benveniste, P*., et al.* Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential. *Cell stem cell* **6**, 48-58 (2010).
6. Notta, F*., et al.* Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. *Science* **333**, 218-221 (2011).
7. Cabezas-Wallscheid, N*., et al.* Identification of regulatory networks in HSCs and their immediate progeny via integrated proteome, transcriptome, and DNA methylome analysis. *Cell stem cell* **15**, 507-522 (2014).
8. Pietras, E.M*., et al.* Functionally Distinct Subsets of Lineage-Biased Multipotent Progenitors Control Blood Production in Normal and Regenerative Conditions. *Cell stem cell* **17**, 35-46 (2015).
9. Oguro, H., Ding, L. & Morrison, S.J. SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell stem cell* **13**, 102-116 (2013).
10. Adolfsson, J*., et al.* Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. *Cell* **121**, 295-306 (2005).
11. Doulatov, S*., et al.* Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nature immunology* **11**, 585-593 (2010).
12. Muller-Sieburg, C.E., Cho, R.H., Thoman, M., Adkins, B. & Sieburg, H.B. Deterministic regulation of hematopoietic stem cell self-renewal and differentiation. *Blood* **100**, 1302-1309 (2002).
13. Sanjuan-Pla, A*., et al.* Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature* **502**, 232-236 (2013).
14. Yamamoto, R*., et al.* Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* **154**, 1112-1126 (2013).
15. Perie, L., Duffy, K.R., Kok, L., de Boer, R.J. & Schumacher, T.N. The Branching Point in Erythro-Myeloid Differentiation. *Cell* **163**, 1655-1662 (2015).
16. Laurenti, E. & Gottgens, B. From haematopoietic stem cells to complex differentiation landscapes. *Nature* **553**, 418-426 (2018).
17. Macaulay, I.C*., et al.* Single-Cell RNA-Sequencing Reveals a Continuous Spectrum of Differentiation in Hematopoietic Cells. *Cell reports* **14**, 966-977 (2016).
18. Nestorowa, S*., et al.* A single-cell resolution map of mouse hematopoietic stem and progenitor cell differentiation. *Blood* **128**, e20-31 (2016).
19. Velten, L*., et al.* Human haematopoietic stem cell lineage commitment is a continuous process. *Nature cell biology* **19**, 271-281 (2017).
20. Tusi, B.K*., et al.* Population snapshots predict early haematopoietic and erythroid hierarchies. *Nature* **555**, 54-60 (2018).
21. Benz, C*., et al.* Hematopoietic stem cell subtypes expand differentially during development and display distinct lymphopoietic programs. *Cell stem cell* **10**, 273-283 (2012).

22. Naik, S.H.*, et al.* Diverse and heritable lineage imprinting of early haematopoietic progenitors. *Nature* **496**, 229-232 (2013).
23. Crane, G.M., Jeffery, E. & Morrison, S.J. Adult haematopoietic stem cell niches. *Nature reviews. Immunology* **17**, 573-590 (2017).
24. Ding, L. & Morrison, S.J. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* **495**, 231-235 (2013).
25. Greenbaum, A.*, et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* **495**, 227-230 (2013).
26. Dykstra, B.*, et al.* Long-term propagation of distinct hematopoietic differentiation programs in vivo. *Cell stem cell* **1**, 218-229 (2007).
27. Morita, Y., Ema, H. & Nakauchi, H. Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *The Journal of experimental medicine* **207**, 1173-1182 (2010).
28. Wilson, A.*, et al.* Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* **135**, 1118-1129 (2008).
29. Cabezas-Wallscheid, N.*, et al.* Vitamin A-Retinoic Acid Signaling Regulates Hematopoietic Stem Cell Dormancy. *Cell* **169**, 807-823 e819 (2017).
30. Simsek, T.*, et al.* The distinct metabolic profile of hematopoietic stem cells reflects their location in a hypoxic niche. *Cell stem cell* **7**, 380-390 (2010).
31. Takubo, K.*, et al.* Regulation of glycolysis by Pdk functions as a metabolic checkpoint for cell cycle quiescence in hematopoietic stem cells. *Cell stem cell* **12**, 49-61 (2013).
32. Ito, K. & Suda, T. Metabolic requirements for the maintenance of self-renewing stem cells. *Nature reviews. Molecular cell biology* **15**, 243-256 (2014).
33. Metcalf, D. Hematopoietic cytokines. *Blood* **111**, 485-491 (2008).
34. Rieger, M.A., Hoppe, P.S., Smejkal, B.M., Eitelhuber, A.C. & Schroeder, T. Hematopoietic cytokines can instruct lineage choice. *Science* **325**, 217-218 (2009).
35. Mossadegh-Keller, N.*, et al.* M-CSF instructs myeloid lineage fate in single haematopoietic stem cells. *Nature* **497**, 239-243 (2013).
36. Muller-Sieburg, C.E., Cho, R.H., Karlsson, L., Huang, J.F. & Sieburg, H.B. Myeloid-biased hematopoietic stem cells have extensive self-renewal capacity but generate diminished lymphoid progeny with impaired IL-7 responsiveness. *Blood* **103**, 4111-4118 (2004).
37. Challen, G.A., Boles, N.C., Chambers, S.M. & Goodell, M.A. Distinct hematopoietic stem cell subtypes are differentially regulated by TGF-beta1. *Cell stem cell* **6**, 265-278 (2010).
38. Heissig, B.*, et al.* The plasminogen fibrinolytic pathway is required for hematopoietic regeneration. *Cell stem cell* **1**, 658-670 (2007).
39. Uckelmann, H.*, et al.* Extracellular matrix protein Matrilin-4 regulates stress-induced HSC proliferation via CXCR4. *The Journal of experimental medicine* **213**, 1961-1971 (2016).
40. Crisan, M. & Dzierzak, E. The many faces of hematopoietic stem cell heterogeneity. *Development* **143**, 4571-4581 (2016).
41. Beerman, I.*, et al.* Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 5465-5470 (2010).
42. Gekas, C. & Graf, T. CD41 expression marks myeloid-biased adult hematopoietic stem cells and increases with age. *Blood* **121**, 4463-4472 (2013).

43. Grover, A.*, et al.* Single-cell RNA sequencing reveals molecular and functional platelet bias of aged haematopoietic stem cells. *Nature communications* **7**, 11075 (2016).
44. Walter, D.*, et al.* Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells. *Nature* **520**, 549-552 (2015).
45. de Haan, G. & Lazare, S.S. Aging of hematopoietic stem cells. *Blood* **131**, 479-487 (2018).
46. Maryanovich, M.*, et al.* Adrenergic nerve degeneration in bone marrow drives aging of the hematopoietic stem cell niche. *Nature medicine* **24**, 782-791 (2018).
47. Hidalgo, A., Chilvers, E.R., Summers, C. & Koenderman, L. The Neutrophil Life Cycle. *Trends in immunology* (2019).
48. Borregaard, N. Neutrophils, from marrow to microbes. *Immunity* **33**, 657-670 (2010).
49. Nerlov, C. & Graf, T. PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors. *Genes & development* **12**, 2403-2412 (1998).
50. Iwasaki, H.*, et al.* Distinctive and indispensable roles of PU.1 in maintenance of hematopoietic stem cells and their differentiation. *Blood* **106**, 1590-1600 (2005).
51. Bjerregaard, M.D., Jurlander, J., Klausen, P., Borregaard, N. & Cowland, J.B. The in vivo profile of transcription factors during neutrophil differentiation in human bone marrow. *Blood* **101**, 4322-4332 (2003).
52. Reddy, V.A.*, et al.* Granulocyte inducer C/EBPalpha inactivates the myeloid master regulator PU.1: possible role in lineage commitment decisions. *Blood* **100**, 483-490 (2002).
53. Dahl, R.*, et al.* Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor. *Nature immunology* **4**, 1029-1036 (2003).
54. Zhang, D.E.*, et al.* Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 569-574 (1997).
55. Radomska, H.S.*, et al.* CCAAT/enhancer binding protein alpha is a regulatory switch sufficient for induction of granulocytic development from bipotential myeloid progenitors. *Molecular and cellular biology* **18**, 4301-4314 (1998).
56. Fazi, F.*, et al.* A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. *Cell* **123**, 819-831 (2005).
57. Pulikkan, J.A.*, et al.* Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. *Blood* **115**, 1768-1778 (2010).
58. Karsunky, H.*, et al.* Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. *Nature genetics* **30**, 295-300 (2002).
59. Hock, H.*, et al.* Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation. *Immunity* **18**, 109-120 (2003).
60. Horman, S.R.*, et al.* Gfi1 integrates progenitor versus granulocytic transcriptional programming. *Blood* **113**, 5466-5475 (2009).
61. Laslo, P.*, et al.* Multilineage transcriptional priming and determination of alternate hematopoietic cell fates. *Cell* **126**, 755-766 (2006).
62. Morosetti, R.*, et al.* A novel, myeloid transcription factor, C/EBP epsilon, is upregulated during granulocytic, but not monocytic, differentiation. *Blood* **90**, 2591-2600 (1997).
63. Yamanaka, R.*, et al.* Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 13187-13192 (1997).
64. Klausen, P., Bjerregaard, M.D., Borregaard, N. & Cowland, J.B. End-stage differentiation of neutrophil granulocytes in vivo is accompanied by up-regulation of p27kip1 and down-regulation of CDK2, CDK4, and CDK6. *Journal of leukocyte biology* **75**, 569-578 (2004).

65. Gery, S., Gombart, A.F., Fung, Y.K. & Koeffler, H.P. C/EBPepsilon interacts with retinoblastoma and E2F1 during granulopoiesis. *Blood* **103**, 828-835 (2004).
66. Lekstrom-Himes, J. & Xanthopoulos, K.G. CCAAT/enhancer binding protein epsilon is critical for effective neutrophil-mediated response to inflammatory challenge. *Blood* **93**, 3096-3105 (1999).
67. Gombart, A.F*., et al.* Neutrophil-specific granule deficiency: homozygous recessive inheritance of a frameshift mutation in the gene encoding transcription factor CCAAT/enhancer binding protein--epsilon. *Blood* **97**, 2561-2567 (2001).
68. Hager, M., Cowland, J.B. & Borregaard, N. Neutrophil granules in health and disease. *Journal of internal medicine* **268**, 25-34 (2010).
69. Borregaard, N., Sorensen, O.E. & Theilgaard-Monch, K. Neutrophil granules: a library of innate immunity proteins. *Trends in immunology* **28**, 340-345 (2007).
70. Boettcher, S. & Manz, M.G. Regulation of Inflammation- and Infection-Driven Hematopoiesis. *Trends in immunology* **38**, 345-357 (2017).
71. Baldridge, M.T., King, K.Y., Boles, N.C., Weksberg, D.C. & Goodell, M.A. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. *Nature* **465**, 793-797 (2010).
72. Essers, M.A*., et al.* IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature* **458**, 904-908 (2009).
73. Lapidot, T. & Kollet, O. The essential roles of the chemokine SDF-1 and its receptor CXCR4 in human stem cell homing and repopulation of transplanted immune-deficient NOD/SCID and NOD/SCID/B2m(null) mice. *Leukemia* **16**, 1992-2003 (2002).
74. Eash, K.J., Means, J.M., White, D.W. & Link, D.C. CXCR4 is a key regulator of neutrophil release from the bone marrow under basal and stress granulopoiesis conditions. *Blood* **113**, 4711-4719 (2009).
75. Eash, K.J., Greenbaum, A.M., Gopalan, P.K. & Link, D.C. CXCR2 and CXCR4 antagonistically regulate neutrophil trafficking from murine bone marrow. *The Journal of clinical investigation* **120**, 2423-2431 (2010).
76. Theilgaard-Monch, K*., et al.* The transcriptional program of terminal granulocytic differentiation. *Blood* **105**, 1785-1796 (2005).
77. Christopher, M.J., Liu, F., Hilton, M.J., Long, F. & Link, D.C. Suppression of CXCL12 production by bone marrow osteoblasts is a common and critical pathway for cytokine-induced mobilization. *Blood* **114**, 1331-1339 (2009).
78. Brinkmann, V*., et al.* Neutrophil extracellular traps kill bacteria. *Science* **303**, 1532-1535 (2004).
79. Papayannopoulos, V. & Zychlinsky, A. NETs: a new strategy for using old weapons. *Trends in immunology* **30**, 513-521 (2009).
80. Phillipson, M. & Kubes, P. The neutrophil in vascular inflammation. *Nature medicine* **17**, 1381-1390 (2011).
81. Pillay, J*., et al.* In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. *Blood* **116**, 625-627 (2010).
82. Galli, S.J., Borregaard, N. & Wynn, T.A. Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils. *Nature immunology* **12**, 1035-1044 (2011).
83. Casanova-Acebes, M*., et al.* Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell* **153**, 1025-1035 (2013).
84. Cossio, I., Lucas, D. & Hidalgo, A. Neutrophils as regulators of the hematopoietic niche. *Blood* **133**, 2140-2148 (2019).
85. Inra, C.N*., et al.* A perisinusoidal niche for extramedullary haematopoiesis in the spleen. *Nature* **527**, 466-471 (2015).
86. Morrison, S.J. & Scadden, D.T. The bone marrow niche for haematopoietic stem cells. *Nature* **505**, 327-334 (2014).

87. Caplan, A.I. Mesenchymal stem cells. *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* **9**, 641-650 (1991).

88. Friedenstein, A.J., Chailakhjan, R.K. & Lalykina, K.S. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell and tissue kinetics* **3**, 393-403 (1970).

89. Acar, M.*, et al.* Deep imaging of bone marrow shows non-dividing stem cells are mainly perisinusoidal. *Nature* **526**, 126-130 (2015).

90. Sugiyama, T., Kohara, H., Noda, M. & Nagasawa, T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* **25**, 977-988 (2006).

91. Omatsu, Y.*, et al.* The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity* **33**, 387-399 (2010).

92. Kunisaki, Y.*, et al.* Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* **502**, 637-643 (2013).

93. Khan, J.A.*, et al.* Fetal liver hematopoietic stem cell niches associate with portal vessels. *Science* **351**, 176-180 (2016).

94. Mendez-Ferrer, S.*, et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466**, 829-834 (2010).

95. Ono, N.*, et al.* Vasculature-associated cells expressing nestin in developing bones encompass early cells in the osteoblast and endothelial lineage. *Developmental cell* **29**, 330-339 (2014).

96. Isern, J.*, et al.* The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. *eLife* **3**, e03696 (2014).

97. Zhou, B.O., Yue, R., Murphy, M.M., Peyer, J.G. & Morrison, S.J. Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell stem cell* **15**, 154-168 (2014).

98. Logan, M.*, et al.* Expression of Cre Recombinase in the developing mouse limb bud driven by a Prxl enhancer. *Genesis* **33**, 77-80 (2002).

99. Ding, L., Saunders, T.L., Enikolopov, G. & Morrison, S.J. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* **481**, 457-462 (2012).

100. Nakashima, K.*, et al.* The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* **108**, 17-29 (2002).

101. Liu, Y.*, et al.* Osterix-cre labeled progenitor cells contribute to the formation and maintenance of the bone marrow stroma. *PloS one* **8**, e71318 (2013).

102. Mizoguchi, T.*, et al.* Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. *Developmental cell* **29**, 340-349 (2014).

103. Park, D.*, et al.* Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. *Cell stem cell* **10**, 259-272 (2012).

104. Maes, C.*, et al.* Osteoblast precursors, but not mature osteoblasts, move into developing and fractured bones along with invading blood vessels. *Developmental cell* **19**, 329-344 (2010).

105. Zhou, X.*, et al.* Multiple functions of Osterix are required for bone growth and homeostasis in postnatal mice. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 12919-12924 (2010).

106. Coskun, S.*, et al.* Development of the fetal bone marrow niche and regulation of HSC quiescence and homing ability by emerging osteolineage cells. *Cell reports* **9**, 581-590 (2014).

107. Li, W., Johnson, S.A., Shelley, W.C. & Yoder, M.C. Hematopoietic stem cell repopulating ability can be maintained in vitro by some primary endothelial cells. *Experimental hematology* **32**, 1226-1237 (2004).

108. Butler, J.M., *et al.* Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell stem cell* **6**, 251-264 (2010).

109. Kobayashi, H., *et al.* Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nature cell biology* **12**, 1046-1056 (2010).

110. Yao, L., Yokota, T., Xia, L., Kincade, P.W. & McEver, R.P. Bone marrow dysfunction in mice lacking the cytokine receptor gp130 in endothelial cells. *Blood* **106**, 4093-4101 (2005).

111. Hooper, A.T., *et al.* Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2-mediated regeneration of sinusoidal endothelial cells. *Cell stem cell* **4**, 263-274 (2009).

112. Winkler, I.G., *et al.* Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* **116**, 4815-4828 (2010).

113. Chow, A., *et al.* Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *The Journal of experimental medicine* **208**, 261-271 (2011).

114. Ludin, A., *et al.* Monocytes-macrophages that express alpha-smooth muscle actin preserve primitive hematopoietic cells in the bone marrow. *Nature immunology* **13**, 1072-1082 (2012).

115. Hur, J., *et al.* CD82/KAI1 Maintains the Dormancy of Long-Term Hematopoietic Stem Cells through Interaction with DARC-Expressing Macrophages. *Cell stem cell* **18**, 508-521 (2016).

116. Chen, X., *et al.* Bone Marrow Myeloid Cells Regulate Myeloid-Biased Hematopoietic Stem Cells via a Histamine-Dependent Feedback Loop. *Cell stem cell* **21**, 747-760 e747 (2017).

117. Bruns, I., *et al.* Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nature medicine* **20**, 1315-1320 (2014).

118. Zhao, M., *et al.* Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. *Nature medicine* **20**, 1321-1326 (2014).

119. Zhao, M., *et al.* FGF signaling facilitates postinjury recovery of mouse hematopoietic system. *Blood* **120**, 1831-1842 (2012).

120. Lucas, D., *et al.* Chemotherapy-induced bone marrow nerve injury impairs hematopoietic regeneration. *Nature medicine* **19**, 695-703 (2013).

121. Mendez-Ferrer, S., Lucas, D., Battista, M. & Frenette, P.S. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* **452**, 442-447 (2008).

122. Yamazaki, S., *et al.* Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. *Cell* **147**, 1146-1158 (2011).

123. Thomson, T.M., *et al.* Expression of human nerve growth factor receptor on cells derived from all three germ layers. *Experimental cell research* **174**, 533-539 (1988).

124. Cattoretti, G., Schiro, R., Orazi, A., Soligo, D. & Colombo, M.P. Bone marrow stroma in humans: anti-nerve growth factor receptor antibodies selectively stain reticular cells in vivo and in vitro. *Blood* **81**, 1726-1738 (1993).

125. Jones, E.A., *et al.* Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal stromal cells from human bone marrow. *Cytometry. Part B, Clinical cytometry* **70**, 391-399 (2006).

126. Jones, E., *et al.* Large-scale extraction and characterization of CD271+ multipotential stromal cells from trabecular bone in health and osteoarthritis: implications for bone regeneration strategies based on uncultured or minimally cultured multipotential stromal cells. *Arthritis and rheumatism* **62**, 1944-1954 (2010).

127. Sacchetti, B., *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131**, 324-336 (2007).

128. Tormin, A.*, et al.* CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. *Blood* **117**, 5067-5077 (2011).

129. Mabuchi, Y.*, et al.* LNGFR(+)THY-1(+)VCAM-1(hi+) cells reveal functionally distinct subpopulations in mesenchymal stem cells. *Stem cell reports* **1**, 152-165 (2013).

130. Isern, J.*, et al.* Self-renewing human bone marrow mesenspheres promote hematopoietic stem cell expansion. *Cell reports* **3**, 1714-1724 (2013).

131. Pinho, S.*, et al.* PDGFRalpha and CD51 mark human nestin+ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *The Journal of experimental medicine* **210**, 1351-1367 (2013).

132. Morikawa, S.*, et al.* Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *The Journal of experimental medicine* **206**, 2483-2496 (2009).

133. Boulais, P.E.*, et al.* The Majority of CD45(-) Ter119(-) CD31(-) Bone Marrow Cell Fraction Is of Hematopoietic Origin and Contains Erythroid and Lymphoid Progenitors. *Immunity* **49**, 627-639 e626 (2018).

134. Penninger, J.M., Irie-Sasaki, J., Sasaki, T. & Oliveira-dos-Santos, A.J. CD45: new jobs for an old acquaintance. *Nature immunology* **2**, 389-396 (2001).

135. Kina, T.*, et al.* The monoclonal antibody TER-119 recognizes a molecule associated with glycophorin A and specifically marks the late stages of murine erythroid lineage. *British journal of haematology* **109**, 280-287 (2000).

136. Newman, P.J.*, et al.* PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science* **247**, 1219-1222 (1990).

137. Chan, C.K.*, et al.* Endochondral ossification is required for haematopoietic stem-cell niche formation. *Nature* **457**, 490-494 (2009).

138. Chan, C.K.*, et al.* Clonal precursor of bone, cartilage, and hematopoietic niche stromal cells. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 12643-12648 (2013).

139. Nagasawa, T., Kikutani, H. & Kishimoto, T. Molecular cloning and structure of a pre-B-cell growth-stimulating factor. *Proceedings of the National Academy of Sciences of the United States of America* **91**, 2305-2309 (1994).

140. Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I. & Littman, D.R. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* **393**, 595-599 (1998).

141. Nie, Y., Han, Y.C. & Zou, Y.R. CXCR4 is required for the quiescence of primitive hematopoietic cells. *The Journal of experimental medicine* **205**, 777-783 (2008).

142. Tzeng, Y.S.*, et al.* Loss of Cxcl12/Sdf-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myelosuppression. *Blood* **117**, 429-439 (2011).

143. Ogawa, M.*, et al.* Expression and function of c-kit in hemopoietic progenitor cells. *The Journal of experimental medicine* **174**, 63-71 (1991).

144. Kaushansky, K.*, et al.* Promotion of megakaryocyte progenitor expansion and differentiation by the c-Mpl ligand thrombopoietin. *Nature* **369**, 568-571 (1994).

145. Tokoyoda, K., Egawa, T., Sugiyama, T., Choi, B.I. & Nagasawa, T. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity* **20**, 707-718 (2004).

146. Williams, D.E.*, et al.* Identification of a ligand for the c-kit proto-oncogene. *Cell* **63**, 167-174 (1990).

147. Zsebo, K.M.*, et al.* Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* **63**, 213-224 (1990).

148. Ikuta, K. & Weissman, I.L. Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proceedings of the National Academy of Sciences of the United States of America* **89**, 1502-1506 (1992).

149. Barker, J.E. Sl/Sld hematopoietic progenitors are deficient in situ. *Experimental hematology* **22**, 174-177 (1994).

150. Barker, J.E. Early transplantation to a normal microenvironment prevents the development of Steel hematopoietic stem cell defects. *Experimental hematology* **25**, 542-547 (1997).

151. Wolf, N.S. Dissecting the hematopoietic microenvironment. III. Evidence for a positive short range stimulus for cellular proliferation. *Cell and tissue kinetics* **11**, 335-345 (1978).

152. Luskey, B.D., Rosenblatt, M., Zsebo, K. & Williams, D.A. Stem cell factor, interleukin-3, and interleukin-6 promote retroviral-mediated gene transfer into murine hematopoietic stem cells. *Blood* **80**, 396-402 (1992).

153. Sitnicka, E.*, et al.* The effect of thrombopoietin on the proliferation and differentiation of murine hematopoietic stem cells. *Blood* **87**, 4998-5005 (1996).

154. Kimura, S., Roberts, A.W., Metcalf, D. & Alexander, W.S. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proceedings of the National Academy of Sciences of the United States of America* **95**, 1195-1200 (1998).

155. Qian, H.*, et al.* Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. *Cell stem cell* **1**, 671-684 (2007).

156. Yoshihara, H.*, et al.* Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell stem cell* **1**, 685-697 (2007).

157. de Sauvage, F.J.*, et al.* Stimulation of megakaryocytopoiesis and thrombopoiesis by the c-Mpl ligand. *Nature* **369**, 533-538 (1994).

158. Kaushansky, K.*, et al.* Thrombopoietin, the Mp1 ligand, is essential for full megakaryocyte development. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 3234-3238 (1995).

159. Sungaran, R., Markovic, B. & Chong, B.H. Localization and regulation of thrombopoietin mRNa expression in human kidney, liver, bone marrow, and spleen using in situ hybridization. *Blood* **89**, 101-107 (1997).

160. Goncalves, K.A.*, et al.* Angiogenin Promotes Hematopoietic Regeneration by Dichotomously Regulating Quiescence of Stem and Progenitor Cells. *Cell* **166**, 894-906 (2016).

161. Himburg, H.A.*, et al.* Dickkopf-1 promotes hematopoietic regeneration via direct and niche-mediated mechanisms. *Nature medicine* **23**, 91-99 (2017).

162. Varnum-Finney, B.*, et al.* Notch2 governs the rate of generation of mouse long- and short-term repopulating stem cells. *The Journal of clinical investigation* **121**, 1207-1216 (2011).

163. Calvi, L.M.*, et al.* Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425**, 841-846 (2003).

164. Ruggero, D. & Shimamura, A. Marrow failure: a window into ribosome biology. *Blood* **124**, 2784-2792 (2014).

165. Shwachman, H., Diamond, L.K., Oski, F.A. & Khaw, K.T. The Syndrome of Pancreatic Insufficiency and Bone Marrow Dysfunction. *The Journal of pediatrics* **65**, 645-663 (1964).

166. Bodian, M., Sheldon, W. & Lightwood, R. Congenital Hypoplasia of the Exocrine Pancreas. *Acta paediatrica* **53**, 282-293 (1964).

167. Burke, V., Colebatch, J.H., Anderson, C.M. & Simons, M.J. Association of pancreatic insufficiency and chronic neutropenia in childhood. *Archives of disease in childhood* **42**, 147-157 (1967).

168. Pringle, E.M., Young, W.F. & Haworth, E.M. Syndrome of pancreatic insufficiency, blood dyscrasia and metaphyseal dysplasia. *Proceedings of the Royal Society of Medicine* **61**, 776-778 (1968).

169. Aggett, P.J.*, et al.* Shwachman's syndrome. A review of 21 cases. *Archives of disease in childhood* **55**, 331-347 (1980).

170. Mack, D.R., Forstner, G.G., Wilschanski, M., Freedman, M.H. & Durie, P.R. Shwachman syndrome: exocrine pancreatic dysfunction and variable phenotypic expression. *Gastroenterology* **111**, 1593-1602 (1996).

171. Dror, Y. & Freedman, M.H. Shwachman-Diamond syndrome: An inherited preleukemic bone marrow failure disorder with aberrant hematopoietic progenitors and faulty marrow microenvironment. *Blood* **94**, 3048-3054 (1999).

172. Goobie, S.*, et al.* Shwachman-Diamond syndrome with exocrine pancreatic dysfunction and bone marrow failure maps to the centromeric region of chromosome 7. *American journal of human genetics* **68**, 1048-1054 (2001).

173. Alter, B.P. Diagnosis, genetics, and management of inherited bone marrow failure syndromes. *Hematology. American Society of Hematology. Education Program*, 29-39 (2007).

174. Donadieu, J.*, et al.* Classification of and risk factors for hematologic complications in a French national cohort of 102 patients with Shwachman-Diamond syndrome. *Haematologica* **97**, 1312-1319 (2012).

175. Boocock, G.R.*, et al.* Mutations in SBDS are associated with Shwachman-Diamond syndrome. *Nature genetics* **33**, 97-101 (2003).

176. Kawakami, T.*, et al.* Genetic analysis of Shwachman-Diamond syndrome: phenotypic heterogeneity in patients carrying identical SBDS mutations. *The Tohoku journal of experimental medicine* **206**, 253-259 (2005).

177. Ceci, M.*, et al.* Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. *Nature* **426**, 579-584 (2003).

178. Finch, A.J.*, et al.* Uncoupling of GTP hydrolysis from eIF6 release on the ribosome causes Shwachman-Diamond syndrome. *Genes & development* **25**, 917-929 (2011).

179. Sen, S.*, et al.* The ribosome-related protein, SBDS, is critical for normal erythropoiesis. *Blood* **118**, 6407-6417 (2011).

180. Rawls, A.S., Gregory, A.D., Woloszynek, J.R., Liu, F. & Link, D.C. Lentiviral-mediated RNAi inhibition of Sbds in murine hematopoietic progenitors impairs their hematopoietic potential. *Blood* **110**, 2414-2422 (2007).

181. Tulpule, A.*, et al.* Pluripotent stem cell models of Shwachman-Diamond syndrome reveal a common mechanism for pancreatic and hematopoietic dysfunction. *Cell stem cell* **12**, 727-736 (2013).

182. Zambetti, N.A.*, et al.* Deficiency of the ribosome biogenesis gene Sbds in hematopoietic stem and progenitor cells causes neutropenia in mice by attenuating lineage progression in myelocytes. *Haematologica* **100**, 1285-1293 (2015).

183. Xia, J.*, et al.* Somatic mutations and clonal hematopoiesis in congenital neutropenia. *Blood* **131**, 408-416 (2018).

184. Aul, C., Giagounidis, A. & Germing, U. Epidemiological features of myelodysplastic syndromes: results from regional cancer surveys and hospital-based statistics. *International journal of hematology* **73**, 405-410 (2001).

185. Vardiman, J.W.*, et al.* The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. *Blood* **114**, 937-951 (2009).

186. Tefferi, A. & Vardiman, J.W. Myelodysplastic syndromes. *The New England journal of medicine* **361**, 1872-1885 (2009).

187. Raza, A.*, et al.* Apoptosis in bone marrow biopsy samples involving stromal and hematopoietic cells in 50 patients with myelodysplastic syndromes. *Blood* **86**, 268-276 (1995).

188. Greenberg, P.*, et al.* International scoring system for evaluating prognosis in myelodysplastic syndromes. *Blood* **89**, 2079-2088 (1997).

189. Greenberg, P.L.*, et al.* Revised international prognostic scoring system for myelodysplastic syndromes. *Blood* **120**, 2454-2465 (2012).

190. Park, S.*, et al.* Predictive factors of response and survival in myelodysplastic syndrome treated with erythropoietin and G-CSF: the GFM experience. *Blood* **111**, 574-582 (2008).
191. Fenaux, P. & Ades, L. How we treat lower-risk myelodysplastic syndromes. *Blood* **121**, 4280-4286 (2013).
192. Itzykson, R.*, et al.* Prognostic factors for response and overall survival in 282 patients with higher-risk myelodysplastic syndromes treated with azacitidine. *Blood* **117**, 403-411 (2011).
193. Cutler, C.S.*, et al.* A decision analysis of allogeneic bone marrow transplantation for the myelodysplastic syndromes: delayed transplantation for low-risk myelodysplasia is associated with improved outcome. *Blood* **104**, 579-585 (2004).
194. Zipperer, E.*, et al.* The hematopoietic stem cell transplantation comorbidity index is of prognostic relevance for patients with myelodysplastic syndrome. *Haematologica* **94**, 729-732 (2009).
195. Ades, L., Itzykson, R. & Fenaux, P. Myelodysplastic syndromes. *Lancet* **383**, 2239-2252 (2014).
196. Papaemmanuil, E.*, et al.* Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts. *The New England journal of medicine* **365**, 1384-1395 (2011).
197. Yoshida, K.*, et al.* Frequent pathway mutations of splicing machinery in myelodysplasia. *Nature* **478**, 64-69 (2011).
198. Moran-Crusio, K.*, et al.* Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation. *Cancer cell* **20**, 11-24 (2011).
199. Bejar, R.*, et al.* Clinical effect of point mutations in myelodysplastic syndromes. *The New England journal of medicine* **364**, 2496-2506 (2011).
200. Shih, A.H., Abdel-Wahab, O., Patel, J.P. & Levine, R.L. The role of mutations in epigenetic regulators in myeloid malignancies. *Nature reviews. Cancer* **12**, 599-612 (2012).
201. Dohner, H., Weisdorf, D.J. & Bloomfield, C.D. Acute Myeloid Leukemia. *The New England journal of medicine* **373**, 1136-1152 (2015).
202. Deschler, B. & Lubbert, M. Acute myeloid leukemia: epidemiology and etiology. *Cancer* **107**, 2099-2107 (2006).
203. Dohner, H.*, et al.* Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood* **115**, 453-474 (2010).
204. Kadia, T.M., Ravandi, F., Cortes, J. & Kantarjian, H. Toward Individualized Therapy in Acute Myeloid Leukemia: A Contemporary Review. *JAMA oncology* **1**, 820-828 (2015).
205. Yates, J.W., Wallace, H.J., Jr., Ellison, R.R. & Holland, J.F. Cytosine arabinoside (NSC-63878) and daunorubicin (NSC-83142) therapy in acute nonlymphocytic leukemia. *Cancer chemotherapy reports* **57**, 485-488 (1973).
206. Yates, J.*, et al.* Cytosine arabinoside with daunorubicin or adriamycin for therapy of acute myelocytic leukemia: a CALGB study. *Blood* **60**, 454-462 (1982).
207. Short, N.J., Rytting, M.E. & Cortes, J.E. Acute myeloid leukaemia. *Lancet* **392**, 593-606 (2018).
208. Zuckerman, T., Ganzel, C., Tallman, M.S. & Rowe, J.M. How I treat hematologic emergencies in adults with acute leukemia. *Blood* **120**, 1993-2002 (2012).
209. McNerney, M.E., Godley, L.A. & Le Beau, M.M. Therapy-related myeloid neoplasms: when genetics and environment collide. *Nature reviews. Cancer* **17**, 513-527 (2017).
210. Lane, S.W. & Gilliland, D.G. Leukemia stem cells. *Seminars in cancer biology* **20**, 71-76 (2010).
211. Delhommeau, F.*, et al.* Mutation in TET2 in myeloid cancers. *The New England journal of medicine* **360**, 2289-2301 (2009).
212. Shlush, L.I.*, et al.* Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* **506**, 328-333 (2014).

213. Lindsley, R.C.*, et al.* Acute myeloid leukemia ontogeny is defined by distinct somatic mutations. *Blood* **125**, 1367-1376 (2015).

214. Genovese, G.*, et al.* Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *The New England journal of medicine* **371**, 2477-2487 (2014).

215. Jaiswal, S.*, et al.* Age-related clonal hematopoiesis associated with adverse outcomes. *The New England journal of medicine* **371**, 2488-2498 (2014).

216. Steensma, D.P.*, et al.* Clonal hematopoiesis of indeterminate potential and its distinction from myelodysplastic syndromes. *Blood* **126**, 9-16 (2015).

217. Raaijmakers, M.H. Myelodysplastic syndromes: revisiting the role of the bone marrow microenvironment in disease pathogenesis. *International journal of hematology* **95**, 17-25 (2012).

218. Pronk, E. & Raaijmakers, M. The mesenchymal niche in MDS. *Blood* **133**, 1031-1038 (2019).

219. Raaijmakers, M.H.*, et al.* Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* **464**, 852-857 (2010).

220. Kode, A.*, et al.* Leukaemogenesis induced by an activating beta-catenin mutation in osteoblasts. *Nature* **506**, 240-244 (2014).

221. Dong, L.*, et al.* Leukaemogenic effects of Ptpn11 activating mutations in the stem cell microenvironment. *Nature* **539**, 304-308 (2016).

222. Zhang, B.*, et al.* Altered microenvironmental regulation of leukemic and normal stem cells in chronic myelogenous leukemia. *Cancer cell* **21**, 577-592 (2012).

223. Arranz, L.*, et al.* Neuropathy of haematopoietic stem cell niche is essential for myeloproliferative neoplasms. *Nature* **512**, 78-81 (2014).

224. Schepers, K.*, et al.* Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. *Cell stem cell* **13**, 285-299 (2013).

225. Hanoun, M.*, et al.* Acute myelogenous leukemia-induced sympathetic neuropathy promotes malignancy in an altered hematopoietic stem cell niche. *Cell stem cell* **15**, 365-375 (2014).

226. Medyouf, H.*, et al.* Myelodysplastic cells in patients reprogram mesenchymal stromal cells to establish a transplantable stem cell niche disease unit. *Cell stem cell* **14**, 824-837 (2014).

227. Chen, S.*, et al.* Massive parallel RNA sequencing of highly purified mesenchymal elements in low-risk MDS reveals tissue-context-dependent activation of inflammatory programs. *Leukemia* **30**, 1938-1942 (2016).

2

MESENCHYMAL INFLAMMATION DRIVES GENOTOXIC STRESS IN HEMATOPOIETIC STEM CELLS AND PREDICTS DISEASE EVOLUTION IN HUMAN PRE-LEUKEMIA

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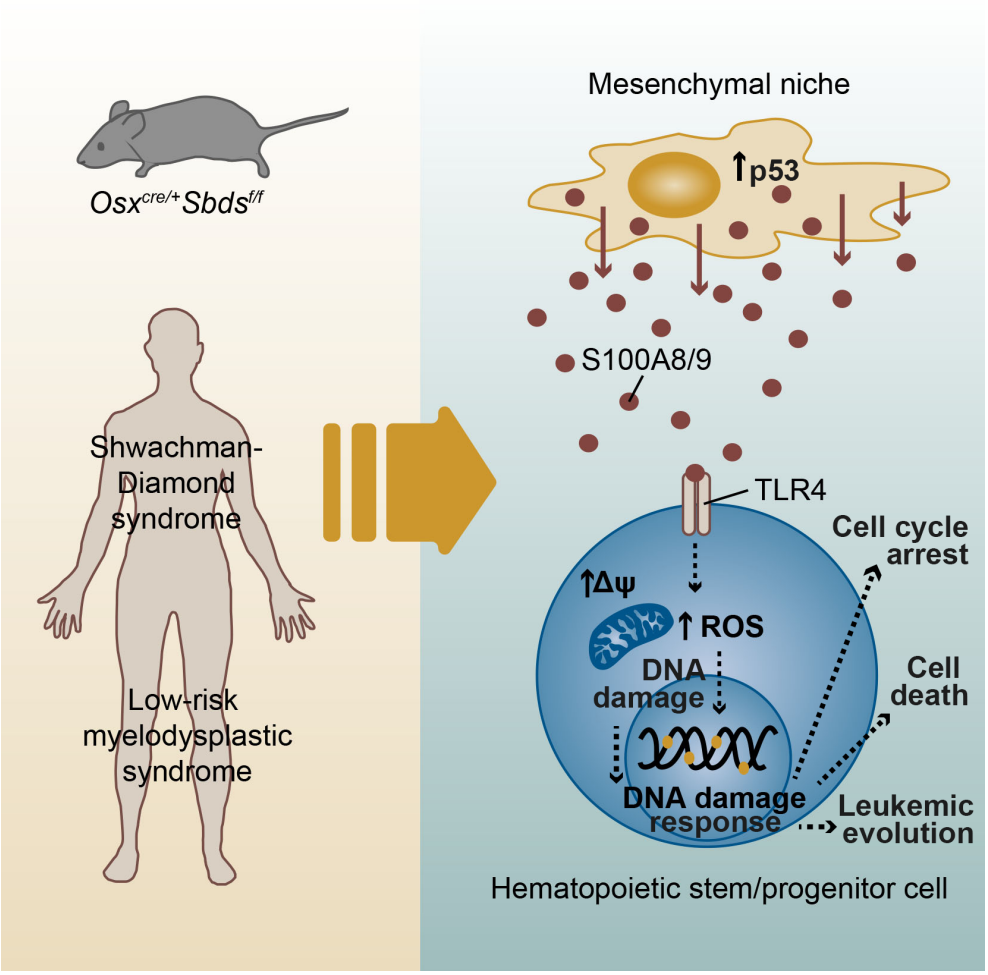


HIGHLIGHTS

- Mesenchymal deletion of *Sbds* in mice recapitulates bone defects in SDS
- Mesenchymal niche cells induce genotoxic stress in HSPCs in this model
- P53-S100A8/9-TLR4 signaling, activated in SDS and MDS, drives these phenotypes
- Mesenchymal S100A8/9 predicts leukemic evolution and disease outcome in human MDS

SUMMARY

Mesenchymal niche cells may drive tissue failure and malignant transformation in the hematopoietic system but the underlying molecular mechanisms and relevance to human disease remain poorly defined. Here, we show that perturbation of mesenchymal cells in a mouse model of the preleukemic disorder Shwachman-Diamond syndrome (SDS) induces mitochondrial dysfunction, oxidative stress and activation of DNA damage responses in hematopoietic stem and progenitor cells. Massive parallel RNA sequencing of highly purified mesenchymal cells in the SDS mouse model and a range of human preleukemic syndromes identified p53-S100A8/9-TLR inflammatory signaling as a common driving mechanism of genotoxic stress. Transcriptional activation of this signaling axis in the mesenchymal niche predicted leukemic evolution and progression-free survival in myelodysplastic syndrome (MDS), the principal leukemia predisposition syndrome. Collectively, our findings identify mesenchymal niche-induced genotoxic stress in heterotypic stem and progenitor cells through inflammatory signaling as a targetable determinant of disease outcome in human preleukemia.

INTRODUCTION

Genotoxic stress results in the accumulation of DNA lesions in hematopoietic stem and progenitor cells (HSPCs) over the lifespan of an organism, contributing to tissue failure and malignant transformation (Jaiswal et al., 2014; Rossi et al., 2007). The pathophysiological insults underlying genomic stress in HSPCs, however, remain incompletely understood. Perturbed signaling from their surrounding microenvironment may be implicated, but this has not been experimentally defined.

Components of the bone marrow microenvironment have emerged as key regulators of normal and malignant hematopoiesis (Arranz et al., 2014; Hanoun et al., 2014; Medyouf et al., 2014; Schepers et al., 2015; Walkley et al., 2007). We, and others, have shown that primary alterations of the mesenchymal niche can induce myelodysplasia and promote the emergence of acute myeloid leukemia (AML) with cytogenetic abnormalities in HSPCs (Kode et al., 2014; Raaijmakers et al., 2010), thus introducing a concept of niche-driven oncogenesis in the hematopoietic system.

To provide insights into the mechanisms that underlie this concept, as well as their relevance for human disease, we modeled the human leukemia predisposition disorder Shwachman-Diamond syndrome (SDS), caused by constitutive homozygous or compound heterozygous loss of function mutations in the *SBDS* gene, required for ribosome biogenesis (Boocock et al., 2003; Finch et al., 2011). SDS is characterized by skeletal defects in conjunction with a striking propensity to develop myelodysplastic syndrome (MDS) and AML at a young age, with a cumulative probability of >30% at the age of 30 years and a median onset at 18 years (Alter, 2007; Donadieu et al., 2012). Hematopoietic cell intrinsic loss of *Sbds* does not result in MDS or leukemia (Rawls et al., 2007; Zambetti et al., 2015), supporting the notion that cell-extrinsic factors contribute to malignant transformation. Deletion of *Sbds* from mesenchymal cells expressing the mesenchymal progenitor marker osterix (*Sp7*) in the bone marrow induced apoptosis in HSPCs and myelodysplasia, but the molecular mechanisms driving these observations and their relevance for human disease remained to be defined (Raaijmakers et al., 2010).

Here, we identify the endogenous damage-associated molecular pattern (DAMP) molecules S100A8 and S100A9, secreted from mesenchymal niche cells, as drivers of mitochondrial dysfunction, oxidative stress and DNA damage response (DDR) activation in HSPCs, with clinical relevance to the pathogenesis and prognosis of human bone marrow failure and leukemia predisposition syndromes.

RESULTS

Deletion of *Sbds* from mesenchymal progenitor cells (MPCs) recapitulates skeletal abnormalities of human SDS

SDS is characterized by bone abnormalities including low-turnover osteoporosis with reduced trabecular bone volume, low numbers of osteoblasts, and reduced amount of osteoid, leading to increased risk of fractures (Toiviainen-Salo et al., 2007). The cellular subsets driving these abnormalities and the underlying molecular mechanisms have remained largely undefined. We have previously shown that Cre-mediated deletion of *Sbds* from osterix^{+} MPCs (*Sbds*$^{f/f}$ *Osx*$^{cre/+}$ mice, hereafter OCS$^{f/f}$ or mutants) disrupts the architecture of the marrow and cortical bone (Raaijmakers et al., 2010). Here, we first sought to better define the skeletal defects in these mice and their relevance to human disease.

OCS$^{f/f}$ mice presented growth retardation and reduced femur length compared to control *Sbds*$^{f/+}$ *Osx*$^{cre/+}$ (OCS$^{f/+}$) mice (Figure 1A and 1B) as observed in human patients (Aggett et al., 1980; Ginzberg et al., 1999). The runted phenotype was associated with a significantly limited lifespan, with lethality observed after the age of 4 weeks. Analyses were therefore performed in three week-old mice. The femur trabecular area was profoundly reduced in OCS$^{f/f}$ mice, with decreased bone volume, low number of trabeculae, increased trabecular spacing and reduced numbers of osteoblasts compared to controls (Figure 1C-1G, and 1I). The cortical bone of OCS mutants was also affected, as indicated by low bone mineral density values (Figure 1C-1D and 1H), attenuating the mechanical properties of the bone, which was found less resistant to fracture in three-point bending test (Figure 1J). A tendency for reduced stiffness in the long bones was also observed (Figure 1K). Taken together, the structural and mechanical defects indicate that *Sbds* deficiency in MPCs causes osteoporosis with a propensity for fracturing, in line with observations in SDS patients (Ginzberg et al., 1999; Mäkitie et al., 2004; Toiviainen-Salo et al., 2007). Impaired osteogenesis did not reflect a contraction of the bone progenitor cell pool as shown by frequency of CFU-F and Osx::GFP^{+} cells (Figure S1A and S1B), but rather impairment of terminal osteogenic differentiation as suggested by transcriptional profiling of prospectively isolated osterix-expressing (GFP^{+}) cells (Figure S1C). Transcriptional data confirmed deregulated expression of genes related to ribosomal biogenesis and translation (Figure S1D and S1E), in line with the established role of *Sbds* in ribosome biogenesis. Collectively, this data supports a view in which bone abnormalities in SDS are caused by deficiency of *Sbds* in MPCs, which attenuates terminal differentiation towards matrix-depositing osteoblastic cells with a compensatory increase in the most primitive mesenchymal compartment.

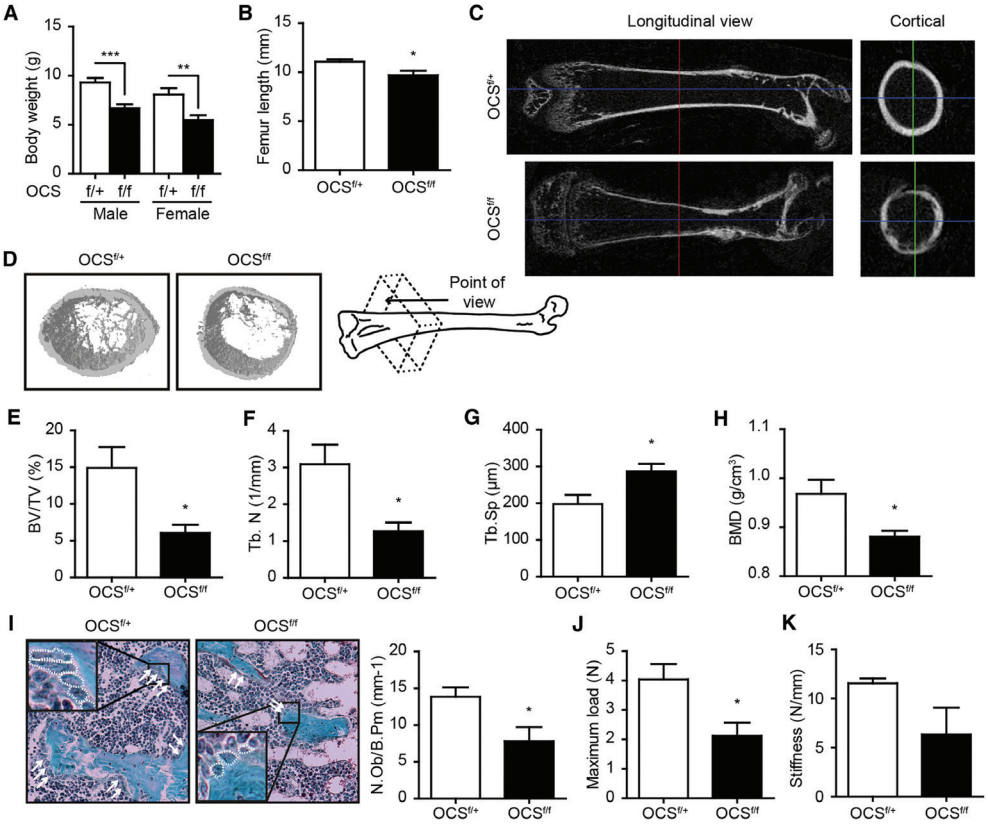


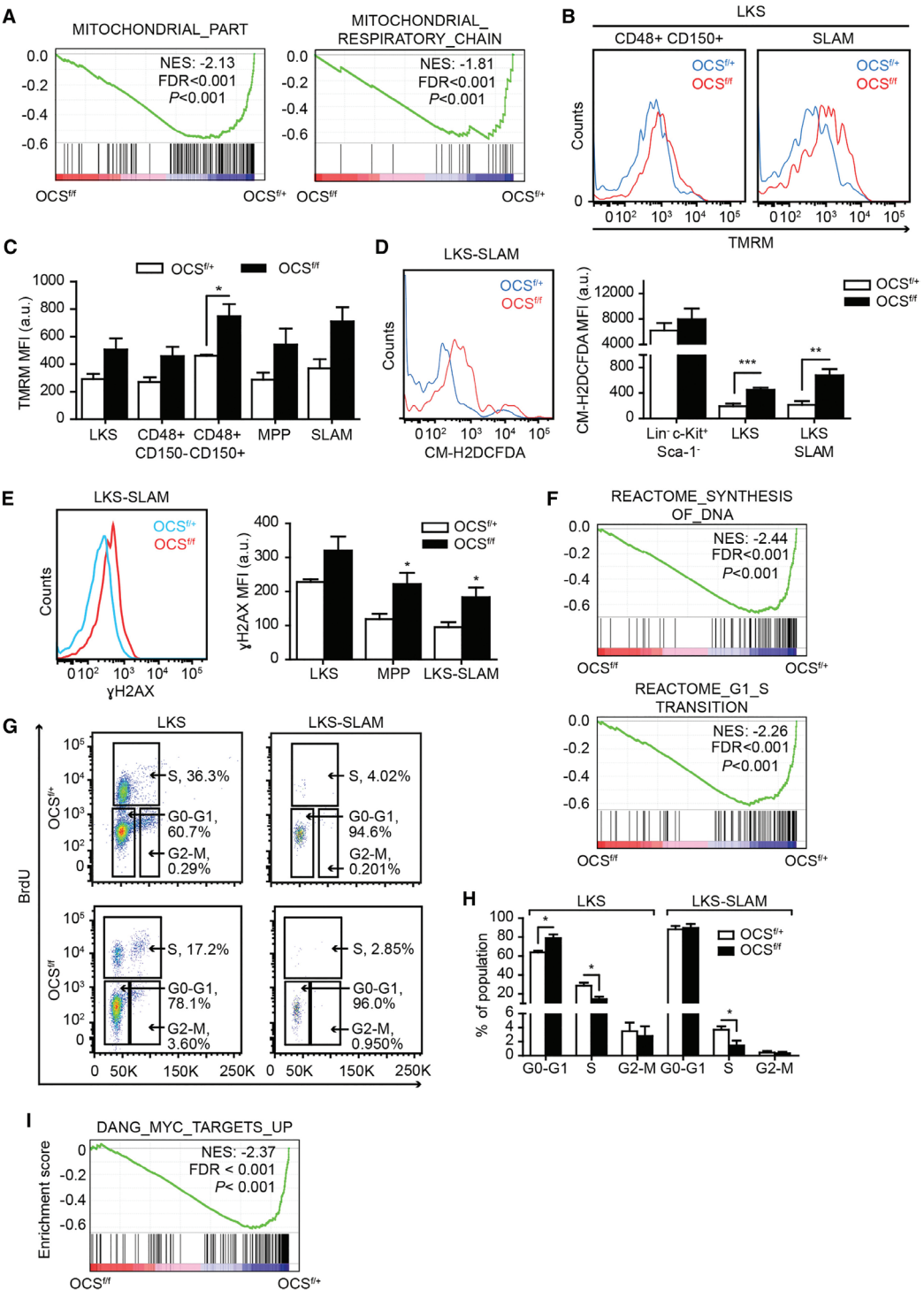
Figure 1. Deletion of *Sbds* in MPCs recapitulates skeletal defects in human SDS. (A-B) Impaired growth in $OCS^{f/f}$ mice: (A) body weight (n = 9) and (B) femur length (n = 5). (C-H) Femur µCT analysis of $OCS^{f/+}$ (n = 5) and $OCS^{f/f}$ (n = 4) mice. (C) Representative 2D-images. Left: longitudinal view. Right: cortical bone. (D) 3D-image. (E) Bone volume per tissue volume (BV/TV). (F) Trabecular number (Tb. N). (G) Trabecular spacing (Tb. Sp). (H) Cortical bone mineral density (BMD). (I) Goldner osteoblast staining ($OCS^{f/+}$, n = 6; $OCS^{f/f}$, n = 8). Left: representative images (arrows: osteoblasts; with white dashed line in the magnified region). Right: number of osteoblasts per bone perimeter (N.Ob/B.Pm). (J-K) 3-point bending test indicating (J) reduced resistant to fracture and (K) decreased stiffness of $OCS^{f/f}$ bone ($OCS^{f/+}$, n = 5; $OCS^{f/f}$, n = 4). *P<0.05. **P< 0.01. ***P<0.001. Data are mean ± SEM. See also Figure S1.

Sbds deficiency in the hematopoietic niche induces mitochondrial dysfunction, oxidative stress and activation of the DNA damage response in HSPCs

Having established that the OCS mice represent a *bona fide* model for bone abnormalities in human disease, we next investigated the hematopoietic consequences of these environmental alterations. HSPC number was unaltered in OCS mice (Figure S2A-S2C) and HSPCs displayed global preservation of their transcriptional landscape after exposure to the *Sbds*-deficient environment (Figure S2D-S2F). Transcriptional network analysis, however, revealed significant overlap with signatures previously defined as predicting leukemic evolution of human $CD34^+$ cells (Li et al., 2011), including pathways signaling mitochondrial abnormalities (Figure 2A; Table S1). Mitochondrial dysfunction was confirmed

by measuring the mitochondrial membrane potential (Δψ), indicating hyperpolarization of the mitochondria (Figure 2B and 2C). Mitochondrial hyperpolarization can result in reverse electron transfer, leading to the production of superoxide radicals, which can be further converted into other reactive oxygen species (ROS) (Murphy, 2009). In line with this, a marked increase in intracellular ROS levels was found in OCS mutant HSPCs (Figure 2D), more specifically superoxide radicals derived from mitochondria as shown by dihydroethidium (DHE) staining (Figure S2G) (Owusu-Ansah et al., 2008; Stowe and Camara, 2009). ROS can undermine the genomic integrity of HSPCs by inducing DNA damage (Ito et al., 2006; Walter et al., 2015; Yahata et al., 2011), to which normal HSPCs react by activating the DDR and DNA repair pathways (Rossi et al., 2007). Indeed, HSPCs (LKS-SLAM) cells from $OCS^{f/f}$ mice displayed accumulation of Ser139-phosphorylated H2AX histone (γH2AX), which forms at the sites of DNA damage (Figure 2E; Figure S2H). Treatment of OCS mutant animals with the ROS scavenger N-acetylcysteine (NAC) resulted in partial reduction in the accumulation of gH2AX (Figure S2I and S2J). Congruent with genotoxic effects of the mutant microenvironment, HSPCs displayed transcriptional modulation of DDR and DNA repair pathways (Table S2), including nucleotide excision repair programs, associated with ROS-induced lesions (Curtin, 2012) and signatures related to the master regulator of DDR and cell checkpoint activation ataxia telangiectasia and Rad3-related (ATR). Activation of the G1-S cell cycle checkpoint, resulting in cell cycle arrest, was suggested by depletion of S-phase transcriptional signatures (Figure 2F; Table S1), *in vivo* BrdU/Ki67 labeling (Figure 2G and 2H; Figure S2K) and downregulation of the Myc pathway, a critical regulator for this restriction point and the coordination of S–G2–M progression (Figure 2I; Table S3). Apoptosis of mutant HSPCs, as an alternative outcome of checkpoint activation, was earlier demonstrated (Raaijmakers et al., 2010). Together, the data indicate that the *Sbds*-deficient environment induces mitochondrial dysfunction, oxidative stress, DNA damage and genotoxic stress in HSPCs leading to activation of DDR pathways and G1-S checkpoint activation, reminiscent of a model in which mitochondrial dysfunction underlies an escalating cycle of increased ROS and genotoxic damage (Sahin and Depinho, 2010).

Short term exposure to the genotoxic environment did not attenuate HSPC function in DNA repair proficient cells, as demonstrated by competitive transplantation experiments (Figure S3A-S3C), suggesting efficient DNA-repair or elimination of functionally impaired HSPCs by the DDR-driven apoptosis and cell cycle arrest. Congruent with this notion, alkaline comet assays on sorted HSPCs failed to demonstrate structural DNA damage (Figure S3D and S3E).


A
MITOCHONDRIAL_PART
NES: -2.13
FDR<0.001
P<0.001
MITOCHONDRIAL_
RESPIRATORY_CHAIN
NES: -1.81
FDR<0.001
P<0.001
B
LKS
CD48+ CD150+
SLAM
Counts
TMRM
C
TMRM MFI (a.u.)
LKS
CD48+ CD150-
CD48+ CD150+
MPP
SLAM
D
LKS-SLAM
Counts
CM-H2DCFDA
CM-H2DCFDA MFI (a.u.)
Lin- c-Kit+ Sca-1-
LKS
LKS SLAM
E
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γH2AX
γH2AX MFI (a.u.)
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F
REACTOME_SYNTHESIS
OF_DNA
NES: -2.44
FDR<0.001
P<0.001
REACTOME_G1_S
TRANSITION
NES: -2.26
FDR<0.001
P<0.001
G
LKS
LKS-SLAM
BrdU
S, 36.3%
G0-G1, 60.7%
G2-M, 0.29%
S, 4.02%
G0-G1, 94.6%
G2-M, 0.201%
S, 17.2%
G0-G1, 78.1%
G2-M, 3,60%
S, 2.85%
G0-G1, 96.0%
G2-M, 0.950%
H
LKS
LKS-SLAM
% of population
G0-G1
S
G2-M
I
DANG_MYC_TARGETS_UP
NES: -2.37
FDR < 0.001
P< 0.001
Enrichment score

Activation of the p53 pathway drives bone abnormalities and genotoxic stress in OCS mice

Next, we sought to define the molecular programs underlying the bone and hematopoietic alterations in OCS mice. A proposed common molecular mechanism for the pathogenesis of ribosomopathies involves activation of the p53 tumor suppressor pathway (Raiser et al., 2014). The p53 protein was overexpressed in GFP^+ MPCs in OCS mutants, with activation of downstream transcriptional pathways and upregulation of canonical targets (Figure 3A-3C). To assess the pathophysiological role of p53 activation in MPCs, we intercrossed OCS with *Trp53*-floxed mice (Marino et al., 2000), generating a double conditional knock-out model where the deletion of p53 is localized in the *Sbds*-deleted stromal compartment (*Sbds*$^{f/f}$ *Trp53*$^{f/f}$ *Osx*$^{cre/+}$ mice; hence OCS$^{f/f}$ p53$^{\Delta}$) (Figure 3D). Genetic recombination of the *Trp53* locus was detected only in bone cells-containing samples, demonstrating the tissue specificity of p53 deletion in this model (Figure 3E). Genetic deletion of p53 from *Sbds*-deficient MPCs rescued the osteoporotic phenotype (Figure 3F-3J), but not cortical bone mineralization (Figure 3K), while it had only modest effects on bone mass in OCS control mice (Figure S4), in line with earlier observations (Wang et al., 2006). Rescue of the skeletal phenotype was linked to amelioration of genotoxic stress in HSPCs as demonstrated by a reduction of superoxide radicals derived from mitochondria and DNA damage (Figure 3L and 3M).

Figure 2. *Sbds*-deficient mesenchymal cells induce genotoxic stress in HSPCs. (A) Transcriptional network analysis indicating mitochondrial dysregulation in mutant HSPCs. NES: Normalized Enrichment Score. (B-C) Increased mitochondrial potential (TMRM) in HSPCs: (B) representative plots; (C) mean fluorescence intensity (MFI) (n = 3). (D) ROS quantification by CM-H2DCFDA (OCS$^{f/+}$, n = 6; OCS$^{f/f}$, n = 7). (E) Increased γH2AX levels in mutant HSPCs. Left, representative FACS analysis. Right, MFI values (n = 4). (F-I) Activation of DNA damage response in mutant HSPCs. (F) Transcriptional repression of G1-S checkpoint progression. (G, H) *In vivo* BrdU staining confirming impaired S-phase transition (n = 4). (I) Downregulation of Myc signaling. GSEA data shown is from $CD48^-$ LKS cells. a.u., arbitrary units. †††FDR<0.001. *P<0.05. **P<0.01. ***P<0.001. Data in bar graphs are mean± SEM. See also Figure S2, S3, S6 and Table S1-S3.

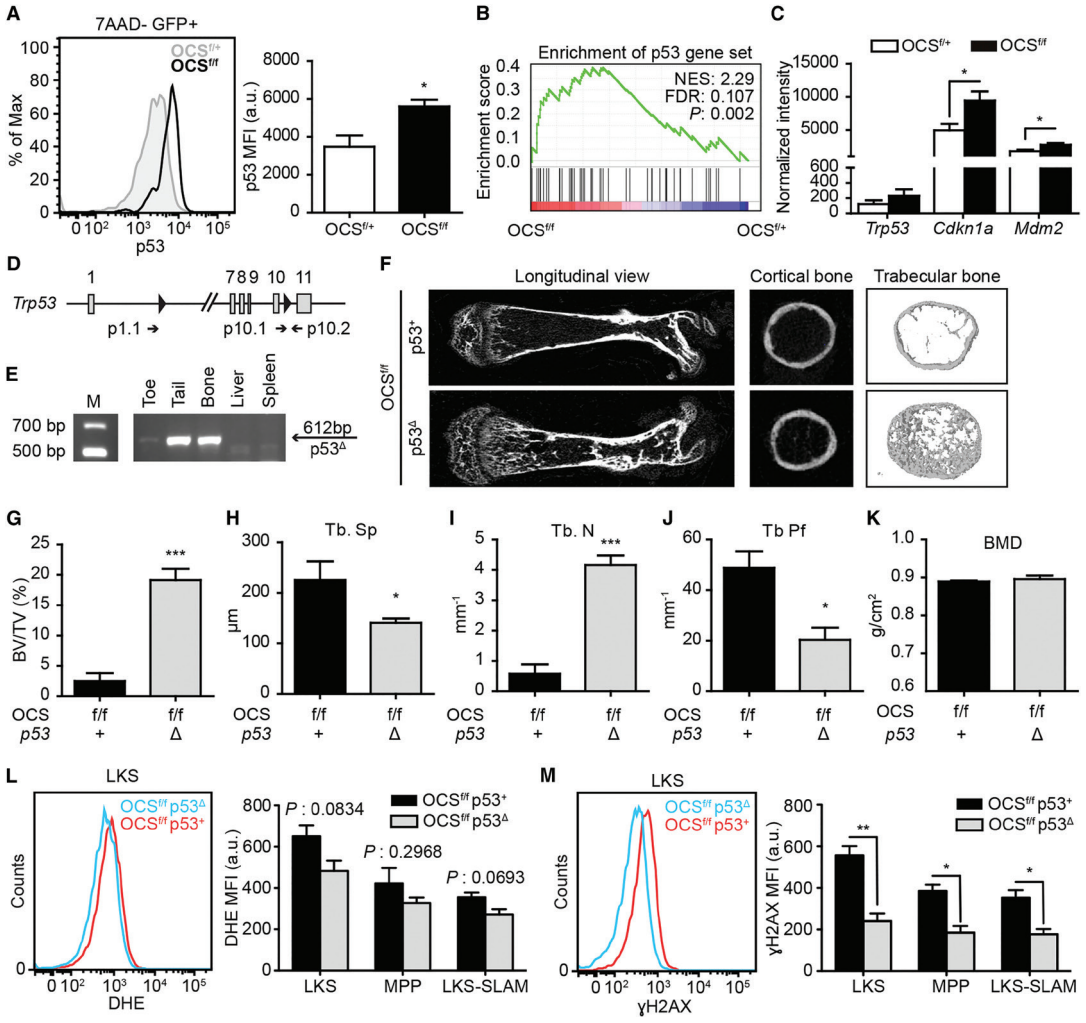


Figure 3. Activation of p53 in MPCs drives skeletal and hematopoietic abnormalities in OCS mutants. (A) p53 protein (FACS) accumulates in GFP$^+$ cells from OCS$^{f/f}$ mice (n = 3). (B-C) Activation of p53 in mutant GFP$^+$ cells as demonstrated by (B) enrichment of a p53 GSEA signature and (C) overexpression of canonical p53 targets (n = 3). (D) Schematic representation of the p53 floxed allele with indication of primers used to assess genotypes (p10.1-p10.2) and genomic deletion (p1.1-p10.2). (E) Specific deletion of p53 in bone-containing tissue in OCS$^{f/f}$ p53$^{\Delta}$ mice (genomic PCR). (F-J) Non-compiled µCT images indicating normalization of bone mass in OCS$^{f/f}$ mice upon genetic deletion of p53 (p53$^+$, n = 3; p53$^{\Delta}$, n = 5). Tb. Pf , Trabecular bone pattern factor. (K) Bone imineral density in OCS$^{f/f}$ mice is not rescued by p53 deletion. (L-M) Effects of p53 deficiency on OCS HSPCs. Tendency for reduced oxidative stress as assessed by DHE analysis (L) and significant reduction of γH2AX levels (M) in HSPCs from OCS$^{f/f}$ p53$^{\Delta}$ mice (n = 3). MFI: mean fluorescence intensity. a.u., arbitrary units. *P < 0.05. **P <0.01. ***P <0.001. Data are mean ± SEM. See also Figure S4.

Identification of the damage-associated molecular pattern genes *S100A8* and *S100A9* as candidate niche factors driving genotoxic stress in human leukemia predisposition syndromes

To identify human disease-relevant niche factors, downstream of p53 activation, driving genomic stress in HSPCs, we compared the transcriptomes of GFP$^+$ MPCs from OCS mice to those from prospectively FACS-isolated mesenchymal CD271$^+$ niche cells (Tormin et al., 2011) from human SDS patients (Figure 4A; Table S4). The mesenchymal nature of CD271$^+$ cells was confirmed by CFU-F capacity and differential expression of mesenchymal, osteolineage and HSPC-regulatory genes (Chen et al, 2016). RNA sequencing showed the presence of *SBDS* mutations (Figure 4B; Figure S5; Table S4) associated with reduced *SBDS* expression (Figure 4C), confirming molecular aspects of SDS in previous studies (Finch et al., 2011; Woloszynek et al., 2004). Virtually identical transcriptional signatures of disrupted ribosome biogenesis and translation were found in human niche cells (Figure 4D) and in GFP$^+$ cells from OCS mice (Figure S1E), confirming faithful recapitulation of human molecular disease characteristics in the mouse model. Forty genes were differentially expressed both in the mouse model and human SDS, 25 of which were overexpressed, with a remarkable abundance of genes encoding proteins implicated in inflammation and innate immunity (Figure 4E).

To further delineate candidate genes driving genomic stress and leukemic evolution from this gene set, we performed whole transcriptome sequencing of CD271$^+$ cells in two related human bone marrow failure and leukemia predisposition disorders: (1) low-risk MDS, the principal human preleukemic disorder in which cell cycle exit (senescence), accumulation of ROS, DNA damage and apoptosis have been described (Head et al., 2011; Peddie et al., 1997; Xiao et al., 2013), reminiscent of HSPC phenotypes in OCS mice, and (2) DBA, like SDS, a ribosomopathy characterized by bone marrow failure, but with a much lower propensity to evolve into AML (<1% with longer latency than observed in SDS and MDS) (Vlachos et al., 2012) (Table S4). We reasoned that genes specifically overexpressed in mesenchymal niche cells from disorders with as strong propensity for leukemic evolution (SDS and MDS) might represent strong candidate drivers of genotoxic stress. Eleven such genes were found (Figure 4F), among which the damage-associated molecular pattern (DAMP) genes *S100A8* and *S100A9*, which were significantly (P<0.05) differentially expressed in GFP$^+$ cells from OCS mutant mice (Figure 4E-4G) and also represent a *bona fide* downstream transcriptional target of p53 (Li et al., 2009). Ex vivo shRNA experiments confirmed that upregulation of both p53 and S100A8/9 are direct, cell-intrinsic consequences of *Sbds* downregulation in mesenchymal precursor (OP9) cells (Figure S6A).

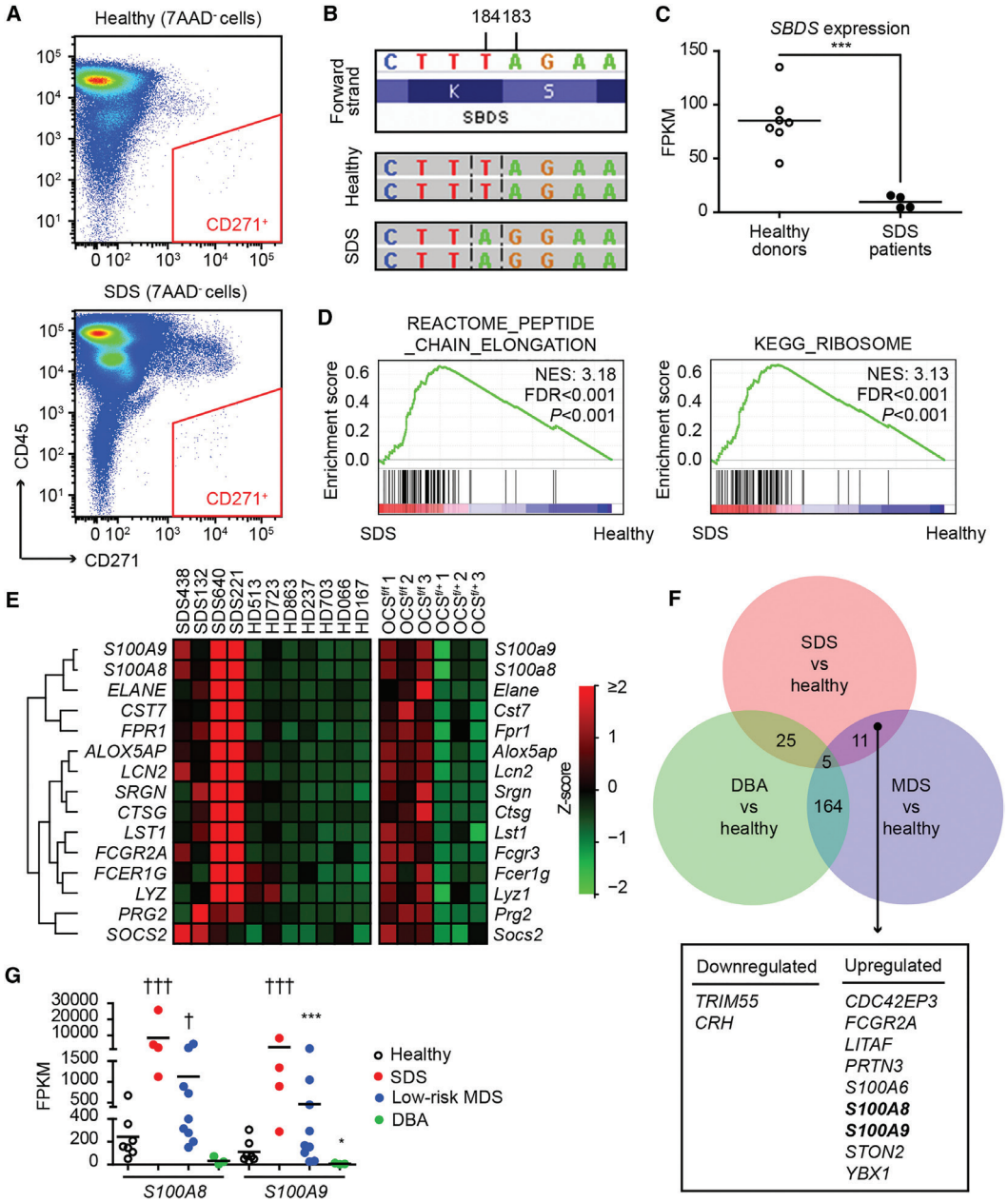


Figure 4. Identification of *S100A8* and *S100A9* as candidate drivers of genotoxic stress in leukemia predisposition syndromes. (A) Representative mesenchymal CD271+ FACS gating. (B) Pathognomonic 183-184 TA>CT mutation in niche cells from a representative SDS patient (IGV plot). (C) Reduced *SBDS* expression in SDS niche cells. (D) Disruption of ribosome biogenesis and translation in SDS CD271+ cells (GSEA). (E) Inflammation-related transcripts are upregulated in niche cells from SDS patients and $OCS^{f/f}$ mice. (F) Significantly differentially expressed genes in SDS (n = 4), MDS (n = 9) and DBA (n = 3) in comparison to normal CD271+ cells. (G) Expression of *S100A8* and *S100A9* in mesenchymal cells from SDS, low-risk MDS, and DBA patients. *P<0.05. ***P<0.001. †††FDR-adjusted P<0.001. See also Figure S1, S5 and Table S4.

Niche-derived S100A8/9 induces genotoxic stress in murine and human HSPCs

S100A8 and *S100A9* belong to a subclass of proinflammatory molecules referred to as DAMP or alarmins. DAMPs are endogenous danger signals that are passively released or actively secreted in the microenvironment after cell death, damage or stress and bind pattern recognition receptors (PRR) to regulate inflammation and tissue repair (Srikrishna and Freeze, 2009). S100A8 and S100A9 proteins were overexpressed in mouse *Sbds*-deficient MPCs (Figure 5A and 5B) and increased plasma concentration of S100A8/9 indicated secretion of the heterodimer (Figure 5C). Its canonical receptor TLR4 (Vogl et al., 2007) is expressed in murine HSPCs (Figure S6B) and the canonical downstream signaling NF-κB and MAPK pathways were activated in HSPCs from $OCS^{f/f}$ mice (Figure S6C).

Exposure of HSPCs (LKS) cells to recombinant murine S100A8/9 resulted in increased DNA damage (number of γH2AX and 53BP1 foci) (Figure 5D; Figure S6D), which was replication-independent (Figure 5E), and apoptosis (Figure 5F), associated with activation of TLR signaling (Figure 5G; Table S5), recapitulating the *in vivo* HSPC phenotype (Raaijmakers et al., 2010). *In vivo*, blockage of TLR4 by neutralizing antibodies resulted in a reduction of γH2AX foci in LKS cells from $OCS^{f/f}$ mice (Figure 5H).

To provide formal experimental support for the view that S100A8/9 production by ancillary cells in the bone marrow microenvironment is sufficient to drive genotoxic stress in HSPCs in a paracrine manner, we next transplanted $CD45.1^{+}$ wild type hematopoietic cells into S100A9-GFP transgenic (S100A9Tg) mice, overexpressing both *S100A8* and *S100A9* under control of the MHC class I H2K promoter (Cheng et al., 2008) (Figure 6A). S100A8/9 (GFP) was expressed in a mesenchymal ($CD45^{-}$ $CD31^{-}$ $Ter119^{-}$ $CD51^{+}$ $Sca1^{-}$) niche population, previously shown to contain the Osterix-expressing cells (Schepers et al, 2013) (Figure 6B and 6C). The $S100A8/9^{+}$ microenvironment induced accumulation of superoxide radicals (DHE) and DNA-damage (γH2AX) in wild type ($CD45.1^{+}$) HSPCs (Figure 6D-6F), in particular in immunophenotypic HSCs, indicating that secretion of S100A8/9 from ancillary cells in the microenvironment is indeed sufficient to induce genotoxic stress in HSCs in a paracrine manner.

Translating these findings to human disease, exposure of human cord blood $CD34^{+}$ HSPCs to human recombinant S100A8/9 at clinically relevant concentrations (Chen et al., 2013 and Supplemental Experimental Procedures) resulted in DNA damage (increased γH2AX foci), apoptosis and impaired HSPC function (CFU-C) (Figure S7).

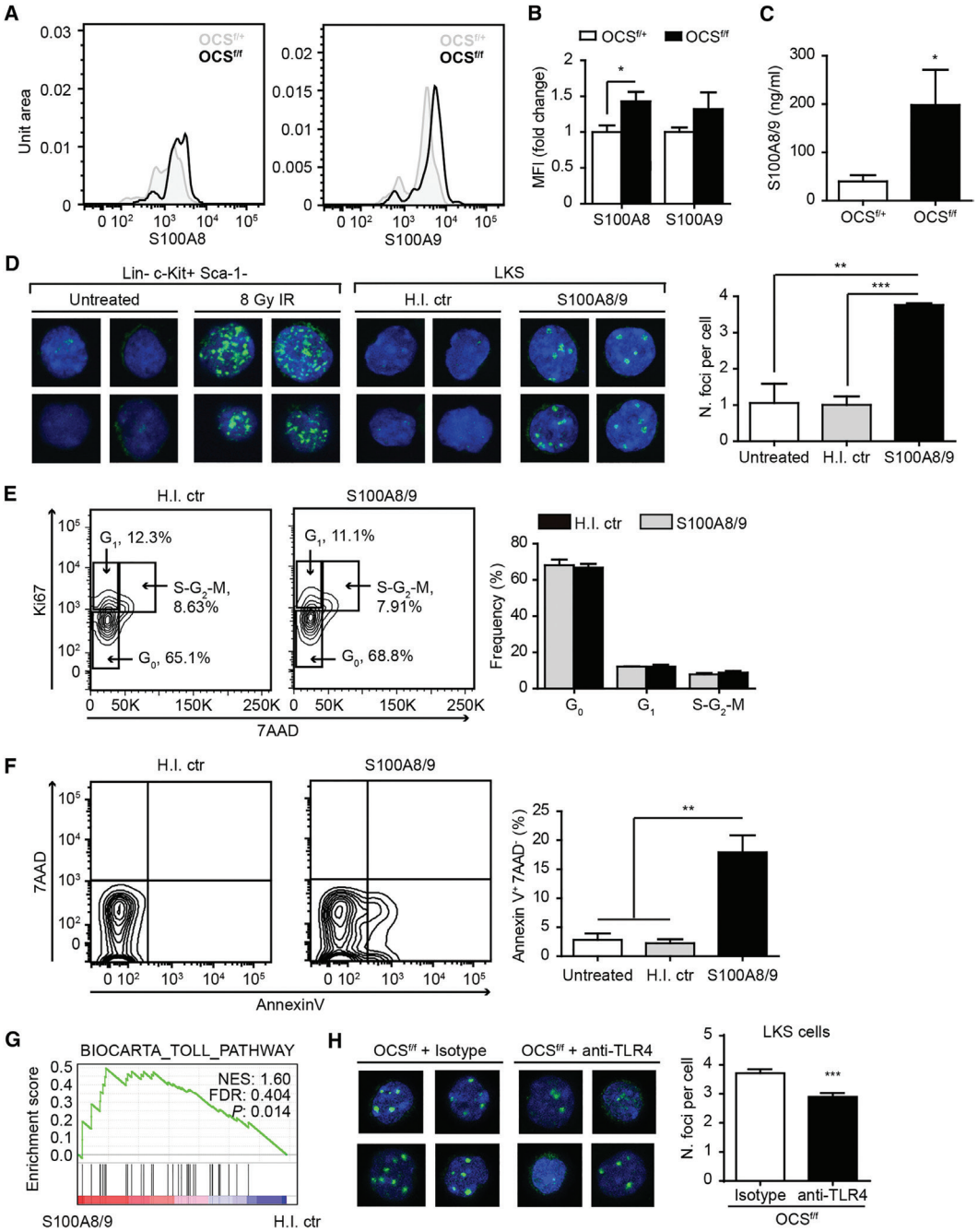


Figure 5. S100A8/9 induces genotoxic stress in murine HSPCs through TLR4 signaling. (A-B) Increased S100A8 and S100A9 levels in OCS$^{f/f}$ GFP^{+} cells. (A) representative plots. (B) MFI values (n = 5). (C) Increased plasma concentration of S100A8/9 by ELISA (OCS$^{f/+}$, n = 5; OCS$^{f/f}$, n = 4). (D) Left: representative γH2AX pictures after HSPCs in vitro exposure. Positive control: 8-Gy irradiated Lin^{-} c-Kit^{+} Sca-1^{-} cells. Negative controls: heat-inactivated S100A8/9 (H.I. ctr). Right: number of γH2AX foci (n = 3). (E) S100A8/9 has no effect on cell cycle (n = 2). (F) Increased apoptosis in S100A8/9-exposed LKS (n = 3). (G) Activation of TLR signaling (GSEA). (H) TLR4-blocking antibodies limit DNA damage in OCS$^{f/f}$ mice (n = 4). *P<0.05. *P<0.01. ***P<0.001. Data are mean ± SEM. See also Figure S6 and S7; Table S5.

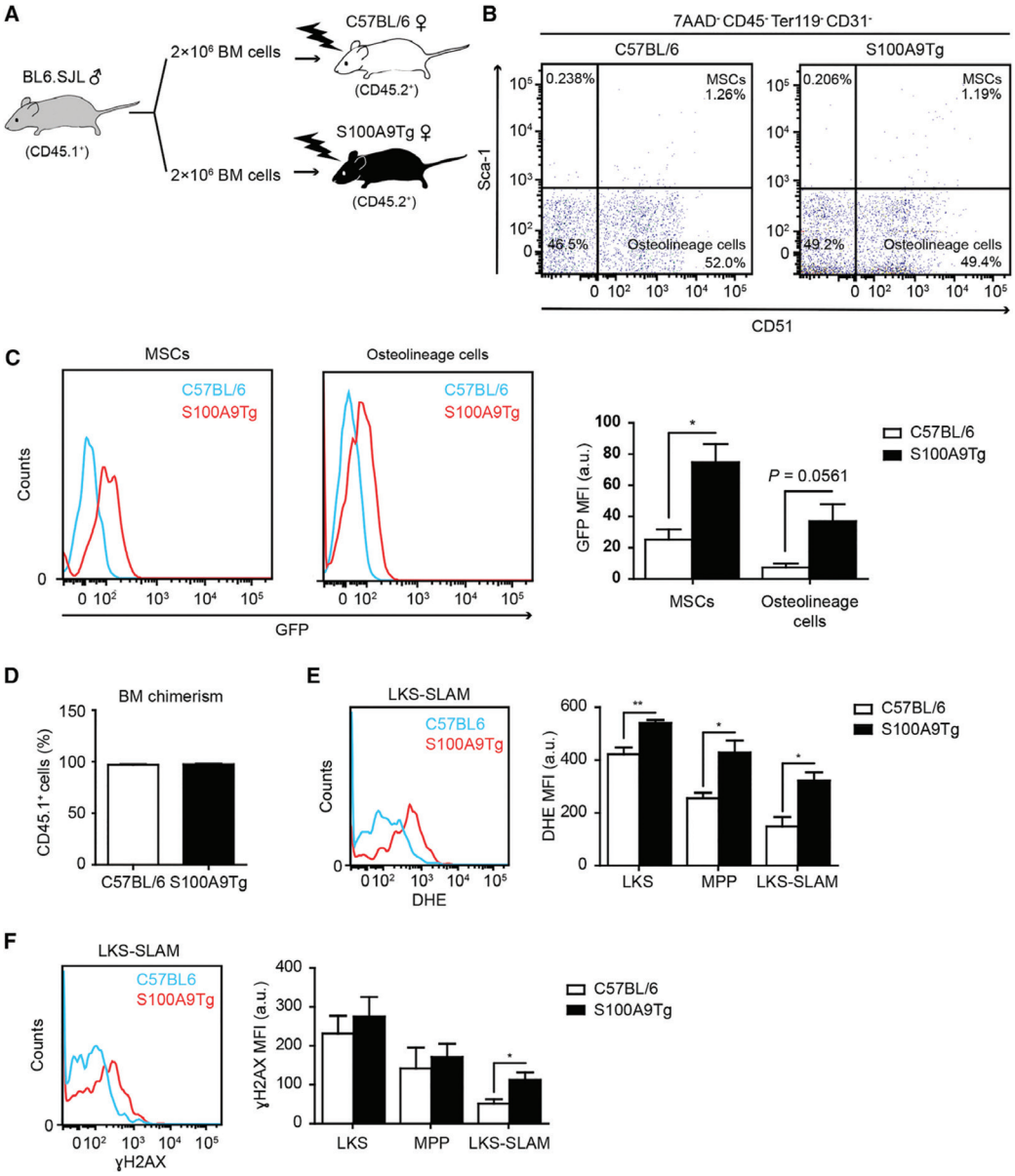
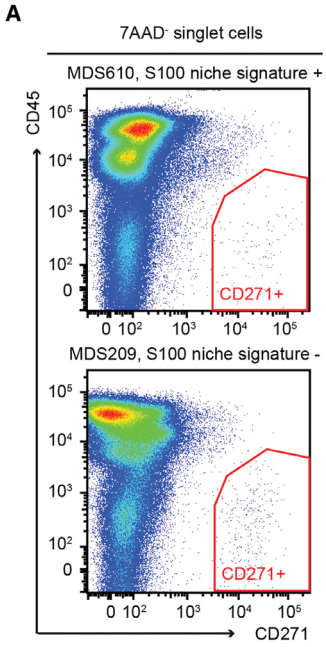


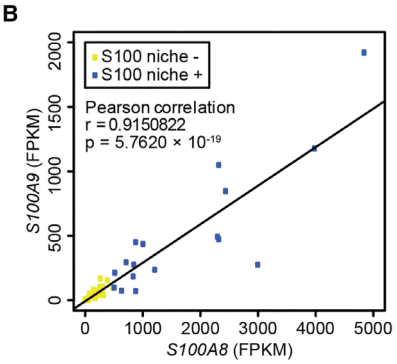
Figure 6. Niche derived S100A8/9 induces oxidative and genotoxic stress in HSPCs. (A) Schematic representation of wild type HSPCs transplantation in S100A9Tg mice. BM: bone marrow. (B-C) Mesenchymal cells from S100A9Tg mice express the S100A9-IRES-GFP construct. (B) Gating strategy defining CD51$^+$ Sca-1$^+$ mesenchymal 'stem' cells (MSC) and CD51$^+$ Sca-1$^-$ osteolineage cells in the microenvironment (C) Expression of GFP in S100A9Tg-derived mesenchymal compartments (n = 3). (D) Transplantation efficiency as assessed by CD45.1$^+$ cell chimerism in the bone marrow (BM) of transplanted mice (n = 4). (E) Accumulation of superoxide radicals in HSPCs exposed to S100A8/9-overexpressing microenvironment. Left, representative plot. Right, DHE MFI values (n = 4). (F) Increased levels of γH2AX in immunophenotypically-defined HSCs. Left, representative plot. Right, DHE MFI values (n = 4). *P<0.05. **P<0.01. Data are mean ± SEM.

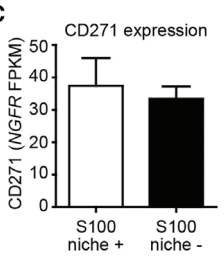
Activation of the p53-S100A8/9-TLR axis in mesenchymal niche cells predicts leukemic evolution and clinical outcome in human low-risk MDS

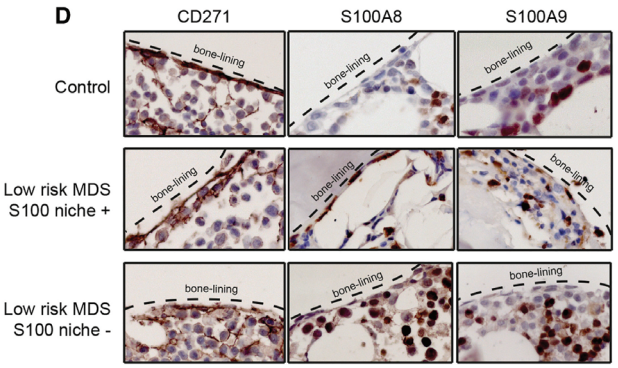
To further define the biologic and clinical significance of these findings, we performed transcriptome sequencing of CD271$^+$ niche cells in a prospective, homogeneously treated cohort of low-risk MDS patients (*n* = 45, Figure 7A; Table S6). Expression of *S100A8* and *S100A9* was strongly correlated (Figure 7B, 7C), with a subgroup of MDS patients (17/45; 38%) demonstrating significant overexpression of *S100A8* and *S100A9* (Modified Thompson Tau outlier test) (Figure 7B and 7D), independent of established prognostic factors as defined by the revised International Prognostic Scoring System (IPSS-R) and the MD Anderson risk score (LR-PSS) (Table S6). Transcriptional pathway analysis (GSEA) comparing mesenchymal cells overexpressing S100A8/9 (*n* = 17) to those of niche S100A8/9$^-$ patients (*n* = 28) revealed activation of p53 and TLR programs in S100A8/9$^+$ mesenchymal cells (Figure 7E), in line with experimental data from the mouse model pointing at the existence of a p53-S100A8/9-TLR axis. Leukemic evolution, defined as the development of frank AML or excess of blasts to WHO RAEB1/ RAEB2 (refractory anemia with excess of blasts), occurred in 5/17 (29.4%) of niche S100A8/9$^+$ patients (3 AML, 2 RAEB1/RAEB2) vs. 4/28 (14.2%) in niche S100A8/9$^-$ patients (2AML, 2 RAEB1/RAEB2). Time to leukemic evolution was significantly shorter in niche S100A8/9$^+$ patients (average 3.4 (1-7.5) vs 18.5 (7-40); *P* = 0.03 by Exact Wilcoxon rank sum test) resulting in a significantly shorter progression-free survival of niche S100A8/9$^+$ patients (median 11.5 vs 53 months, *P* = 0.03) (Figure 7F and 7G). Collectively, the data establish activation of p53-S100A8/9 signaling in mesenchymal niche cells as an independent predictor of disease outcome in human MDS.

Figure 7. Activation of the p53-S100A8/9-TLR axis in mesenchymal niche cells predicts leukemic evolution and clinical outcome in human low-risk MDS. (A) Representative examples of FACS-isolated CD271$^+$ niche cells in human MDS. (B) Correlation plot of *S100A8* and *S100A9* expression levels in human low-risk MDS (*n* = 45). (C) Expression of the defining gene *NGFR* (CD271) in mesenchymal cells (S100 niche +, *n* = 17; S100 niche -, *n* = 28). (D) Representative staining of S100A8 and S100A9 in endosteal (CD271$^+$) stromal cells. Intramedullary staining reflects expression of S100A8/9 in myeloid cells. (E) GSEA analysis indicating enrichment of p53 and TLR signatures in S100A8/9-overexpressing CD271$^+$ cells. Two representative GSEA plots are shown. NES: Normalized Enrichment Score. (F) Kaplan-Meier survival curve showing progression-free survival (G). Statistical analysis indicating significantly reduced time to progression and progression-free survival (PFS). Data are mean ± SEM. See also Table 6.



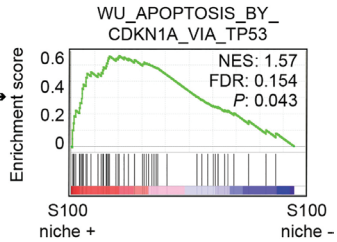


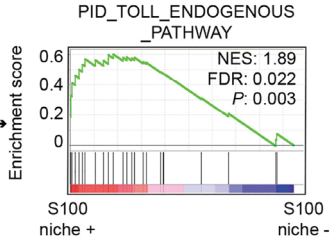


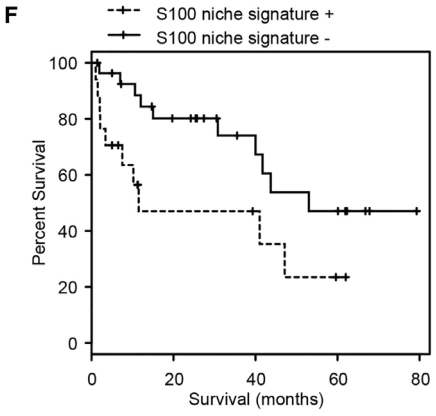


E

Gene set	NES	*P*	FDR
Activation of p53 pathway			
WU_APOPTOSIS_BY_CDKN1A_VIA_TP53	1.57	0.043	0.154
TANG_SENESCENCE_TP53_TARGETS_DN	1.57	0.052	0.156
TANG_SENESCENCE_TP53_TARGETS_UP	1.44	0.055	0.240
Activation of Toll-like receptor pathway			
PID_TOLL_ENDOGENOUS_PATHWAY	1.89	0.003	0.022
BIOCARTA_TOLL_PATHWAY	1.75	0.005	0.069
REACTOME_TOLL_RECEPTOR_CASCADES	1.63	0.008	0.126
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.59	0.010	0.149
REACTOME_ACTIVATED_TLR4_SIGNALLING	1.55	0.026	0.169







G

	Frequency of leukemic evolution	Time to progression (months)	Median PFS (months)
S100 niche signature +	29.4%	3.4	11.5
S100 niche signature -	14.3%	18.5	53
p-value	n.s.	0.03175	0.0301

DISCUSSION

Genomic stress and the ensuing DNA damage play a pivotal role in the attenuation of normal hematopoiesis in ageing and disease. Mutations accumulate in HSPCs over the lifespan of an organism, but the (patho)physiological sources of genomic stress in HSPCs and their relationship with human bone marrow failure remain incompletely understood. Here, we show that specific inflammatory signals from the mesenchymal niche can induce genotoxic stress in heterotypic stem/progenitor cells and relate this concept to the pathogenesis of two human bone marrow failure and leukemia predisposition syndromes, SDS and MDS.

The data indicate that the mesenchymal niche may actively contribute to the formation of a 'mutagenic' environment, adding to our understanding of how a premalignant environment facilitates cancer initiation and evolution. The data argue that this may not only occur through facilitated selection and expansion of genetic clones that stochastically emerge in a permissive environment, but that the mesenchymal niche may be an active participant in driving the genotoxic stress underlying tissue failure and malignant transformation of parenchymal cells.

Notably, leukemic transformation was not observed in mice with targeted deficiency of *Sbds* in mesenchymal cells. Earlier, in a related mouse model of targeted *Dicer1* deletion in MPCs, leukemic transformation was a rare event (Raaijmakers et al., 2010). In the light of our current findings, these observations are likely explained by several factors. First, prolonged exposure to a mutagenic niche, beyond the limited lifespan of OCS mice, may be necessary for the accumulation of genetic damage required for full transformation. Additionally, the data argue that DNA repair-proficient HSPCs are able to cope with the mutagenic stress induced by their environment through activation of the DDR (as shown by molecular activation of cell cycle checkpoints and apoptosis), preventing the accumulation of stable genetic damage (as demonstrated by comet assays) and maintaining the functional integrity of HSPCs (as shown by repopulation assays).

We propose that in SDS (and possibly other congenital bone marrow failure syndromes) genetically aberrant hematopoietic and niche elements cooperate in driving bone marrow failure and leukemic evolution. Our mouse models of SDS support a view in which hematopoietic cell autonomous loss-of-function of *Sbds* drives neutropenia (Zambetti et al., 2015), while niche alterations in this disease drive myelodysplastic alteration and genotoxic stress. It is conceivable that loss-of-function mutations in *Sbds* in HSPCs further sensitizes HSPCs to the genotoxic effects of the *Sbds*-deficient environment, perhaps through attenuation of DNA damage repair mechanisms. It will thus be of considerable interest to test the hypothesis that a mutagenic environment cooperates with aberrant

HSPCs, compromised in their ability to cope with inflammatory genotoxic stress, in leukemia evolution. In this context, the propensity of *Sbds*-deficient cells to accumulate ROS (Ambekar et al., 2010), and their reduced ability to cope with various cellular stressors such as mitotic spindle destabilizing agents, endoplasmic reticulum stress activators, topoisomerase inhibitors and UV irradiation (Austin et al., 2008; Ball et al., 2009) is noteworthy.

The current findings add to emerging insights into the role of innate immune TLR-signaling in the pathogenesis of human MDS. TLR4 and other TLRs are overexpressed in HSPCs from MDS patients (Maratheftis et al., 2007; Wei et al., 2013) and TLR4 expression was shown to correlate with apoptosis in $CD34^+$ hematopoietic cells. TLR signaling is constitutively activated in MDS mice with deletion of chromosome 5 (del5q) (Starczynowski et al., 2010) and multiple TLR downstream signaling pathways have been shown to be activated in MDS and related to loss of progenitor cell function (Ganan-Gomez et al., 2015).

Our findings implicate the DAMP S100A8/9 derived from the mesenchymal niche as a driver of TLR signaling in this disease. The unbiased identification of S100A8/9 seems to independently converge with an earlier report implicating S100A8/9 in the pathogenesis of MDS (Chen et al., 2013). In this study it was shown that the plasma concentration of S100A9 was significantly increased in MDS patients (Chen et al., 2013) and S100A8/9 was shown to drive expansion and activation of myeloid-derived suppressor cells (MDSCs) that contributed to cytopenia and myelodysplasia in a murine model of S100A9 overexpression through secretion of suppressive cytokines. It is therefore an intriguing possibility that additional, indirect, biologic effects of S100A8/9 contribute to the hematopoietic phenotype of OCS mice. This may include engagement of other cognate receptors of the protein, including expansion of MDSCs through CD33 signaling (Chen et al., 2013).

In our study, S100A8/9 was aberrantly overexpressed in a rare population of mesenchymal niche cells, both in the mouse model and human disease. Typically, expression of the protein is found in myeloid cells, raising the question why S100A8/9 production by (rare) niche cells is more relevant to the biology of HSPC than secretion from myeloid/erythroid cells. While the answer to this question remains speculative in the absence of *in vivo* targeted overexpression studies, it is noteworthy that, in contrast to most cytokines, chemokines and other pro-inflammatory molecules, the local accumulation of S100A8/9 in the environment is very high (up to 100µg/ml and about 50 to 100-fold higher than systemic concentrations), likely caused by attachment to extracellular matrices such as proteoglycans (Vogl et al., 2014). This implicates that the exposure of HSPCs to S100A8/9 is projected to relate strongly to their anatomical proximity to a producing cell. $CD271^+$ mesenchymal cells

are directly adjacent to $CD34^+$ HSPC in human bone marrow (Flores-Figueroa et al., 2012). This notion of 'spatial relevance' may also be congruent with recent observations that aberrant overexpression of S100A8/9 in hematopoietic (erythroid) cells within the erythroid island in a model of human $5q^-$ syndrome leads to a predominant erythroid, anemic, phenotype (Schneider at al., 2016).

The mechanisms of S100A8/9 induced DNA damage remain to be fully elucidated. Our experiments using NAC to reduce ROS burden suggest an incomplete association between oxidative stress and DNA damage, suggesting that S100A8/9 secretion may attenuate genomic integrity through additional mechanisms. Similarly, it is conceivable that other ligands secreted from mesenchymal cells contribute to the induction of DNA damage in HSPCs in the mouse model. We found a striking abundance of transcripts encoding other DAMPs and cytotoxic proteins in both the mouse model and mesenchymal elements isolated from SDS patients. Ongoing investigations will have to assess whether other selected ligands can evoke genomic stress in heterotypic HSPCs and in such a fashion contribute to the generation of a mutagenic environment in these disorders.

Finally, our findings establish molecular characteristics of the mesenchymal environment as an important determinant of disease outcome in humans. S100A8/9 expression, associated with activated p53 and TLR signaling, in mesenchymal cells predicted leukemic evolution and progression-free survival in a cohort of homogeneously treated low-risk MDS patients. This is of considerable clinical relevance because low-risk MDS is a heterogeneous disease-entity with a subset of patients having a particular dismal prognosis not identified by current risk-stratification strategies (Bejar et al., 2012). Gene expression of S100A8/9 may identify a substantial subset of patients with a survival typically associated with 'high-risk' patients and, if confirmed in larger independent cohorts, could guide therapeutic decision making in MDS. The data thus provide a strong rationale for niche–instructed therapeutic targeting of inflammatory signaling in human preleukemic disease.

EXPERIMENTAL PROCEDURES

Mice and *in vivo* procedures

OCS, *Trp53*$^{f/f}$ and S100A9Tg mice have been previously described (Cheng et al., 2008; Jonkers et al., 2001; Raaijmakers et al., 2010). *Ptprc*^{a}*Pepc*^{b}*/BoyCrl* (B6.SJL) mice were purchased from Charles River. Animals were maintained in specific pathogen free conditions in the Experimental Animal Center of Erasmus MC (EDC). For *in vivo* cell cycle analysis, OCS mice received intraperitoneal injections of BrdU (1.5 mg in PBS, BD Biosciences) and sacrificed after 15 h. For TLR4 studies, 2 week-old mice were intraperitoneally injected with a double dose (100 µg and 35 µg, 48 h interval) of TLR4-neutralizing antibody (clone MTS510, eBioscience) or isotype control (clone eBR2a, eBioscience) and sacrificed after 60h. For NAC rescue studies, 2-week-old mice received daily intraperitoneal injections of NAC (Sigma-Aldrich, 320 mg/kg in saline) until the day of the analysis and at least for 5 days. All mice were sacrificed by cervical dislocation. Animal studies were approved by the Animal Welfare/ Ethics Committee of the EDC in accordance with legislation in the Netherlands (Approval No. EMC 2067, 2714, 2892, 3062).

µCT analysis

Femur bones were isolated, fixated in 3% PFA/PBS for 24 h and stored in 70% ethanol. µCT analysis was performed using a SkyScan 1172 system (SkyScan) using previously described settings (Tudpor et al., 2015). Bone microarchitectural parameters relative to the trabecular and the cortical area were determined in the distal metaphysis and the mid-diaphysis of each femur, respectively, using software packages from Bruker MicroCT (NRecon, CtAn and Dataviewer).

Human bone marrow samples

Bone marrow aspirates were obtained from SDS and DBA patients during routine follow-up. All MDS patients were treated with lenalidomide (10 mg/day, d 1-21 in a 4-week schedule) in the context of an ongoing prospective clinical trial for patients with low or intermediate-1 risk MDS according to IPSS criteria (HOVON89; www.hovon.nl; www.trialregister.nl as NTR1825; EudraCT No. 2008-002195-10). Bone marrow specimens were collected at study entry and disease diagnosis and staging confirmed by central board reviewing. Leukemic evolution was assessed according to WHO criteria; development of RAEB1 (refractory anemia with excess of blast) or RAEB-2 (if RAEB1 at entry) was considered progression of disease. Leukemia (AML) was diagnosed according to standard WHO criteria (≥20% myeloblasts in blood/bone marrow). Bone marrow cells from allogeneic transplantation donors were used as normal controls. Patients and healthy donor characteristics are described in Table S4 and S6. All specimens were collected with informed consent, in accordance with the Declaration of Helsinki.

Gene expression profiling

Osx::GFP cells from bone cell suspensions of OCS mice were sorted in TRIzol Reagent (Life Technologies) and RNA was extracted according to the manufacturer's recommendations. Linear amplification of mRNA was performed using the Ovation Pico WTA System (NuGEN). cDNA was fragmented and labelled with Encore™ Biotin Module (NuGEN). The biotinylated cDNA was hybridized to the GeneChip Mouse Genome 430 2.0 Array (Affymetrix eBioscience). Signal was normalized and differential gene expression analysis was performed with the limma package (Ritchie et al., 2015). Gene expression array data derived from murine Osterix::GFP cells is available at ArrayExpress (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-5023. RNA sequencing experiments were performed as previously described (Zambetti et al., 2015). Human transcripts were aligned to the Ref Seq transcriptome (hg19) and analyzed with DESeq2 (Love et al., 2014), while mouse transcripts were aligned to the Ensembl transcriptome (mm10) and analyzed with EdgeR (Robinson et al., 2010) in the R environment. FPKM values were calculated using Cufflinks (Trapnell et al., 2010). Principal component analysis was performed in the R environment on the raw fragment counts extracted from the BAM files by HTSeq-count (Anders et al., 2015). RNA-Seq data derived from murine $CD48^{-}/CD48^{+}$ LSK cells have been deposited at the European Nucleotide Archive (ENA, http://www.ebi.ac.uk/ena/), which is hosted by the EBI, under accession number PRJEB15060. RNA-seq data derived from human LR-MDS, SDS and DBA specimens have been deposited at the European Genome-phenome Archive (EGA, https://www.ebi.ac.uk/ega/), which is hosted by the EBI, under accession number EGAS00001001926. For Gene Set Enrichment Analysis (Subramanian et al., 2005) (GSEA, Broad Institute), normalized intensity values (microarray data) and FPKM values (RNA sequencing) were compared to the curated gene sets (C2) and the Gene Ontology gene sets (C5) of the Molecular Signature Database (MsigDB) using the Signal2Noise metric and 1000 gene set-based permutations. For HSPCs GO-term analysis, genes with significantly differential expression ($P<0.05$) were interrogated using g:Profiler web-based software (Reimand et al., 2011; Reimand et al., 2007).

Immunofluorescence microscopy

HSPCs were harvested in PBS+0.5%FBS, cytospun on a glass slide for 3 min at 500 rpm using a Cytospin 4 centrifuge (Thermo Scientific) and fixed in 3% PFA/PBS for 15 min on ice. After 3 washing steps in PBS, cells were permeabilized for 2 min in 0.15% Triton-X100/PBS. Aspecific binding sites were blocked by incubation in 1%BSA/PBS for 1 h at room temperature. Cells were next stained overnight at 4°C with either anti-phospho-histone H2A.X (Ser139) mouse monoclonal antibody (clone JBW301, Merck Millipore, diluted 1:1000 in 1%BSA/PBS) or with anti-53BP1 rabbit polyclonal antibody (Novus Biologicals, diluted 1:1000 in 1%BSA/PBS). Slides were washed twice in PBS for 5 min and incubated for 1 h at 37°C with either Alexa Fluor 488-conjugated goat anti-mouse antibody (Cat. A10667, Life Technologies) or

goat anti-rabbit antibody (Cat. A11008, Life Technologies), both diluted 1:200 in 1%BSA/PBS. After 2 washes in PBS, slides were mounted in VECTASHIELD Mounting Medium with DAPI (Vector Laboratories). Z-series images were acquired with a Leica TCS SP5 confocal microscope (63X objective lens) using the LAS software (Leica Microsystems). γH2AX and 53BP1 foci were counted manually from the maximum projection view.

Survival analysis

The low-risk MDS patient subgroup with S100 niche signature was defined by the Modified Thompson Tau test for outlier detection. In brief, *S100A8* statistics from the control cases were combined to define the rejection region, demarcating FPKM values to be considered as outliers. MDS cases with *S100A8* FPKM values within the rejection region were thus defined as niche-signature^{+}. To determine the significance difference in time to progression we used the Wilcoxon signed rank test accounting for tied observations. Event-free survival was determined by specifying leukemic progression or death as events. Patients experiencing a non-hematological related death (e.g., cardiac failure), where censored on the date of this event. Patients remaining alive were censored on the date of last consultation. Kaplan-Meier curves were used to estimate the survival functions through time. Statistical differences in the survival distributions was assessed with the Mantel-Cox log-rank test. All calculations were performed in the R environment.

Statistics

Statistical analysis was performed using Prism 5 (GraphPad Software). Unless otherwise specified, unpaired, 2-tailed Student's t test (single test) or 1-way analysis of variance (multiple comparisons) were used to evaluate statistical significance, defined as $P<0.05$. All results in bar graphs are mean value ± standard error of the mean.

ACCESSION NUMBERS

The accession number for the gene expression array data derived from murine Osterix::GFP cells is ArrayExpress: E-MTAB-5023. The accession number for the RNA-seq data derived from murine CD48/CD48+ LSK cells is European Nucleotide Archive, which is hosted by the EBI: PRJEB15060. The accession number for the RNA-seq data derived from human LR-MDS, SDS, and DBA specimens is European Genome-phenome Archive, which is hosted by the EBI: EGAS00001001926.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and six tables.

AUTHOR CONTRIBUTIONS

Conceptualization: N.A.Z. and M.H.G.P.R.; Methodology: N.A.Z., Z.P., S.C., K.J.G.K., M.A.M., E.M.J.B., B.V.D.E., J.P.T.M.V.L., R.K., T.V., and M.H.G.P.R.; Investigation: N.A.Z., Z.P., S.C., M.A.M., E.M.J.B., M.N.A., P.M.H.V.S., C.V.D.L., B.V.D.E., and T.V.; Resources: T.M.W., E.M.P.C., C. M., P. G. M., J.P.T.M.V.L, I.P.T., T.W.K., R.K., A.V.D.L., and T.V.; Data curation: M.A.S., R.M.H., T.M.W., E.M.P.C., T.W.K., and A.V.D.L.; Writing: N.A.Z. and M.H.G.P.R; Visualization: N.A.Z., Z.P., S.C, and R.H.M. ; Supervision and Funding Acquisition: M.H.G.P.R.

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REFERENCES

Aggett, P.J., Cavanagh, N.P.C., Matthew, D.J., Pincott, J.R., Sutcliffe, J., and Harries, J.T. (1980). Shwachmans Syndrome - a Review of 21 Cases. Arch. Dis. Child. *55*, 331-347.

Alter, B.P. (2007). Diagnosis, genetics, and management of inherited bone marrow failure syndromes. Hematology Am. Soc. Hematol. Educ. Program, 29-39.

Ambekar, C., Das, B., Yeger, H., and Dror, Y. (2010). SBDS-deficiency results in deregulation of reactive oxygen species leading to increased cell death and decreased cell growth. Pediatr. Blood Cancer *55*, 1138-1144.

Anders, S., Pyl, P.T., and Huber, W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. Bioinformatics *31*, 166-169.

Arranz, L., Sanchez-Aguilera, A., Martin-Perez, D., Isern, J., Langa, X., Tzankov, A., Lundberg, P., Muntion, S., Tzeng, Y.S., Lai, D.M.*, et al.* (2014). Neuropathy of haematopoietic stem cell niche is essential for myeloproliferative neoplasms. Nature *512*, 78-81.

Austin, K.M., Gupta, M.L., Jr., Coats, S.A., Tulpule, A., Mostoslavsky, G., Balazs, A.B., Mulligan, R.C., Daley, G., Pellman, D., and Shimamura, A. (2008). Mitotic spindle destabilization and genomic instability in Shwachman-Diamond syndrome. J. Clin. Invest. *118*, 1511-1518.

Ball, H.L., Zhang, B., Riches, J.J., Gandhi, R., Li, J., Rommens, J.M., and Myers, J.S. (2009). Shwachman-Bodian Diamond syndrome is a multi-functional protein implicated in cellular stress responses. Hum. Mol. Genet. *18*, 3684-3695.

Bejar, R., Stevenson, K.E., Caughey, B.A., Abdel-Wahab, O., Steensma, D.P., Galili, N., Raza, A., Kantarjian, H., Levine, R.L., Neuberg, D.*, et al.* (2012). Validation of a prognostic model and the impact of mutations in patients with lower-risk myelodysplastic syndromes. J. Clin. Oncol. *30*, 3376-3382.

Boocock, G.R., Morrison, J.A., Popovic, M., Richards, N., Ellis, L., Durie, P.R., and Rommens, J.M. (2003). Mutations in SBDS are associated with Shwachman-Diamond syndrome. Nat. Genet. *33*, 97-101.

Chen, S., Zambetti, N.A., Bindels, E.M., Kenswill, K., Mylona A.M., Adisty, N.M., Hoogenboezem, R.M., Sanders, M.A., Cremers, E.M., Westers, T.M., *et al*. (2016). Massive parallel RNA sequencing of highly purified mesenchymal elements in low-risk MDS reveals tissue-context-dependent activation of inflammatory programs. Leukemia http://dx.doi.org/10.1038/leu.2016.91.

Chen, X., Eksioglu, E.A., Zhou, J., Zhang, L., Djeu, J., Fortenbery, N., Epling-Burnette, P., Van Bijnen, S., Dolstra, H., Cannon, J.*, et al.* (2013). Induction of myelodysplasia by myeloid-derived suppressor cells. J. Clin. Invest. *123*, 4595-4611.

Cheng, P., Corzo, C.A., Luetteke, N., Yu, B., Nagaraj, S., Bui, M.M., Ortiz, M., Nacken, W., Sorg, C., Vogl, T., et al. (2008). Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. J. Exp. Med. *205*, 2235-2249.

Curtin, N.J. (2012). DNA repair dysregulation from cancer driver to therapeutic target. Nat. Rev. Cancer *12*, 801-817.

Donadieu, J., Fenneteau, O., Beaupain, B., Beaufils, S., Bellanger, F., Mahlaoui, N., Lambilliotte, A., Aladjidi, N., Bertrand, Y., Mialou, V.*, et al.* (2012). Classification of and risk factors for hematologic complications in a French national cohort of 102 patients with Shwachman-Diamond syndrome. Haematologica *97*, 1312-1319.

Finch, A.J., Hilcenko, C., Basse, N., Drynan, L.F., Goyenechea, B., Menne, T.F., Fernandez, A.G., Simpson, P., D'Santos, C.S., Arends, M.J.*, et al.* (2011). Uncoupling of GTP hydrolysis from eIF6 release on the ribosome causes Shwachman-Diamond syndrome. Genes Dev. *25*, 917-929.

Flores-Figueroa, E., Varma, S., Montgomery, K., Greenberg, P.L., Gratzinger, D. (2012). Distinctive contact between CD34+ hematopoietic progenitors and CXCL12+ CD271+ mesenchymal stromal cells in benign and myelodysplastic bone marrow. Lab. Invest. *92*, 1330-1341.

Ganan-Gomez, I., Wei, Y., Starczynowski, D.T., Colla, S., Yang, H., Cabrero-Calvo, M., Bohannan, Z.S., Verma, A., Steidl, U., and Garcia-Manero, G. (2015). Deregulation of innate immune and inflammatory signaling in myelodysplastic syndromes. Leukemia *29*, 1458-1469.

Ginzberg, H., Shin, J., Ellis, L., Morrison, J., Ip, W., Dror, Y., Freedman, M., Heitlinger, L.A., Belt, M.A., Corey, M., *et al.* (1999). Shwachman syndrome: phenotypic manifestations of sibling sets and isolated cases in a large patient cohort are similar. J. Pediatr. *135*, 81-88.

Hanoun, M., Zhang, D., Mizoguchi, T., Pinho, S., Pierce, H., Kunisaki, Y., Lacombe, J., Armstrong, S.A., Duhrsen, U., and Frenette, P.S. (2014). Acute myelogenous leukemia-induced sympathetic neuropathy promotes malignancy in an altered hematopoietic stem cell niche. Cell Stem Cell *15*, 365-375.

Head, D.R., Jacobberger, J.W., Mosse, C., Jagasia, M., Dupont, W., Goodman, S., Flye, L., Shinar, A., McClintock-Treep, S., Stelzer, G., *et al.* (2011). Innovative analyses support a role for DNA damage and an aberrant cell cycle in myelodysplastic syndrome pathogenesis. Bone Marrow Res. *2011*, 950934.

Ito, K., Hirao, A., Arai, F., Takubo, K., Matsuoka, S., Miyamoto, K., Ohmura, M., Naka, K., Hosokawa, K., Ikeda, Y., *et al.* (2006). Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. Nat. Med. *12*, 446-451.

Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P.V., Mar, B.G., Lindsley, R.C., Mermel, C.H., Burtt, N., Chavez, A., *et al.* (2014). Age-related clonal hematopoiesis associated with adverse outcomes. N. Engl. J. Med. *371*, 2488-2498.

Jonkers, J., Meuwissen, R., van der Gulden, H., Peterse, H., van der Valk, M., and Berns, A. (2001). Synergistic tumor suppressor activity of BRCA2 and p53 in a conditional mouse model for breast cancer. Nat. Genet. *29*, 418-425.

Kode, A., Manavalan, J.S., Mosialou, I., Bhagat, G., Rathinam, C.V., Luo, N., Khiabanian, H., Lee, A., Murty, V.V., Friedman, R., *et al.* (2014). Leukaemogenesis induced by an activating beta-catenin mutation in osteoblasts. Nature *506*, 240-244.

Li, C., Chen, H., Ding, F., Zhang, Y., Luo, A., Wang, M., and Liu, Z. (2009). A novel p53 target gene, S100A9, induces p53-dependent cellular apoptosis and mediates the p53 apoptosis pathway. Biochem. J. *422*, 363-372.

Li, L., Li, M., Sun, C., Francisco, L., Chakraborty, S., Sabado, M., McDonald, T., Gyorffy, J., Chang, K., Wang, S., *et al.* (2011). Altered hematopoietic cell gene expression precedes development of therapy-related myelodysplasia/acute myeloid leukemia and identifies patients at risk. Cancer Cell *20*, 591-605.

Love, M.I., Huber, W., and Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. *15*, 550.

Mäkitie, O., Ellis, L., Durie, P.R., Morrison, J.A., Sochett, E.B., Rommens, J.M., and Cole, W.G. (2004). Skeletal phenotype in patients with Shwachman-Diamond syndrome and mutations in SBDS. Clin. Genet. *65*, 101-112.

Maratheftis, C.I., Andreakos, E., Moutsopoulos, H.M., and Voulgarelis, M. (2007). Toll-like receptor-4 is up-regulated in hematopoietic progenitor cells and contributes to increased apoptosis in myelodysplastic syndromes. Clin. Cancer Res. *13*, 1154-1160.

Marino, S., Vooijs, M., van der Gulden, H., Jonkers, J., and Berns, A. (2000). Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the external granular layer cells of the cerebellum. Genes Dev. *14*, 994-1004.

Medyouf, H., Mossner, M., Jann, J.C., Nolte, F., Raffel, S., Herrmann, C., Lier, A., Eisen, C., Nowak, V., Zens, B., *et al.* (2014). Myelodysplastic cells in patients reprogram mesenchymal stromal cells to establish a transplantable stem cell niche disease unit. Cell Stem Cell *14*, 824-837.

Murphy, M.P. (2009). How mitochondria produce reactive oxygen species. Biochem. J. *417*, 1-13.

Owusu-Ansah, E., Yavari, A., Mandal, S., and Banerjee, U. (2008). Distinct mitochondrial retrograde signals control the G1-S cell cycle checkpoint. Nat Genet. *40*, 356-361.

Peddie, C.M., Wolf, C.R., McLellan, L.I., Collins, A.R., and Bowen, D.T. (1997). Oxidative DNA damage in CD34+ myelodysplastic cells is associated with intracellular redox changes and elevated plasma tumour necrosis factor-alpha concentration. Br. J. Haematol. *99*, 625-631.

Raaijmakers, M.H., Mukherjee, S., Guo, S., Zhang, S., Kobayashi, T., Schoonmaker, J.A., Ebert, B.L., Al-Shahrour, F., Hasserjian, R.P., Scadden, E.O*., et al.* (2010). Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. Nature *464*, 852-857.

Raiser, D.M., Narla, A., and Ebert, B.L. (2014). The emerging importance of ribosomal dysfunction in the pathogenesis of hematologic disorders. Leuk. Lymphoma *55*, 491-500.

Rawls, A.S., Gregory, A.D., Woloszynek, J.R., Liu, F.L., and Link, D.C. (2007). Lentiviral-mediated RNAi inhibition of Sbds in murine hematopoietic progenitors impairs their hematopoietic potential. Blood *110*, 2414-2422.

Reimand, J., Arak, T., and Vilo, J. (2011). g:Profiler--a web server for functional interpretation of gene lists (2011 update). Nucleic Acids Res. *39*, W307-315.

Reimand, J., Kull, M., Peterson, H., Hansen, J., and Vilo, J. (2007). g:Profiler--a web-based toolset for functional profiling of gene lists from large-scale experiments. Nucleic Acids Res. *35*, W193-200.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. *43*, e47.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics *26*, 139-140.

Rossi, D.J., Bryder, D., Seita, J., Nussenzweig, A., Hoeijmakers, J., and Weissman, I.L. (2007). Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. Nature *447*, 725-729.

Sahin, E., and Depinho, R.A. (2010). Linking functional decline of telomeres, mitochondria and stem cells during ageing. Nature *464*, 520-528.

Schepers, K., Campbell, T.B., and Passegue, E. (2015). Normal and leukemic stem cell niches: insights and therapeutic opportunities. Cell Stem Cell *16*, 254-267.

Schepers, K., Pietras, E.M., Reynaud, D., Flach, J., Binnewies, M., Garg, T., Wagers, A.J., Hsiao, E.C., and Passegué, E. (2013). Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. Cell Stem Cell *13*, 285-299.

Schneider, R.K., Schenone, M., Ferreira, M.V., Kramann, R., Joyce, C.E., Hartigan, C., Beier, F., Brümmendorf, T.H., Germing, U., Platzbecker, U. *et al.* (2016). Rps14 haploinsufficiency causes a block in erythroid differentiation mediated by S100A8 and S100A9. Nat. Med. *22*, 288-297.

Srikrishna, G., and Freeze, H.H. (2009). Endogenous damage-associated molecular pattern molecules at the crossroads of inflammation and cancer. Neoplasia *11*, 615-628.

Stowe, D.F., and Camara, A.K.S. (2009). Mitochondrial Reactive Oxygen Species Production in Excitable Cells: Modulators of Mitochondrial and Cell Function. Antioxid. Redox Signal. *11*, 1373-1414.

Subramanian, A., Tamayo, P., Mootha, V.K., Mukherjee, S., Ebert, B.L., Gillette, M.A., Paulovich, A., Pomeroy, S.L., Golub, T.R., Lander, E.S*., et al.* (2005). Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl. Acad. Sci. USA *102*, 15545-15550.

Toiviainen-Salo, S., Mayranpaa, M.K., Durie, P.R., Richards, N., Grynpas, M., Ellis, L., Ikegawa, S., Cole, W.G., Rommens, J., Marttinen, E*., et al.* (2007). Shwachman-Diamond syndrome is associated with low-turnover osteoporosis. Bone *41*, 965-972.

Tormin, A., Li, O., Brune, J.C., Walsh, S., Schutz, B., Ehinger, M., Ditzel, N., Kassem, M., and Scheding, S. (2011). CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. Blood *117*, 5067-5077.

Starczynowski, D.T., Kuchenbauer, F., Argiropoulos, B., Sung, S., Morin, R., Muranyi, A., Hirst, M., Hogge, D., Marra, M., Wells, R.A., *et al*. (2010). Identification of miR-145 and miR-146a as mediators of the 5q- syndrome phenotype. Nat. Med. *16*, 49-58.

Trapnell, C., Williams, B.A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M.J., Salzberg, S.L., Wold, B.J., and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. *28*, 511-515.

Tudpor, K., van der Eerden, B.C., Jongwattanapisan, P., Roelofs, J.J., van Leeuwen, J.P., Bindels, R.J., and Hoenderop, J.G. (2015). Thrombin receptor deficiency leads to a high bone mass phenotype by decreasing the RANKL/OPG ratio. Bone *72*, 14-22.

Vlachos, A., Rosenberg, P.S., Atsidaftos, E., Alter, B.P., and Lipton, J.M. (2012). Incidence of neoplasia in Diamond Blackfan anemia: a report from the Diamond Blackfan Anemia Registry. Blood *119*, 3815-3819.

Vogl, T., Eisenblätter, M., Völler, T., Zenker, S., Hermann, S., van Lent, P., Faust, A., Geyer, C., Petersen, B., Roebrock, K., *et al*. (2014). Alarmin S100A8/S100A9 as a biomarker for molecular imaging of local inflammatory activity. Nat. Commun. *6*,4593.

Vogl, T., Tenbrock, K., Ludwig, S., Leukert, N., Ehrhardt, C., van Zoelen, M.A., Nacken, W., Foell, D., van der Poll, T., Sorg, C*., et al.* (2007). Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nat. Med. *13*, 1042-1049.

Walkley, C.R., Olsen, G.H., Dworkin, S., Fabb, S.A., Swann, J., McArthur, G.A., Westmoreland, S.V., Chambon, P., Scadden, D.T., and Purton, L.E. (2007). A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. Cell *129*, 1097-1110.

Walter, D., Lier, A., Geiselhart, A., Thalheimer, F.B., Huntscha, S., Sobotta, M.C., Moehrle, B., Brocks, D., Bayindir, I., Kaschutnig, P*., et al.* (2015). Exit from dormancy provokes DNA-damage-induced attrition in haematopoietic stem cells. Nature *520*, 549-552.

Wang, X., Kua, H.Y., Hu, Y., Guo, K., Zeng, Q., Wu, Q., Ng, H.H., Karsenty, G., de Crombrugghe, D., Yeh, J., *et al.* (2006). p53 functions as a negative regulator of osteoblastogenesis, osteoblast-dependent osteoclastogenesis, and bone remodeling. J. Cell. Biol. *172*, 115-125.

Wei, Y., Dimicoli, S., Bueso-Ramos, C., Chen, R., Yang, H., Neuberg, D., Pierce, S., Jia, Y., Zheng, H., Wang, H*., et al.* (2013). Toll-like receptor alterations in myelodysplastic syndrome. Leukemia *27*, 1832-1840.

Woloszynek, J.R., Rothbaum, R.J., Rawls, A.S., Minx, P.J., Wilson, R.K., Mason, P.J., Bessler, M., and Link, D.C. (2004). Mutations of the SBDS gene are present in most patients with Shwachman-Diamond syndrome. Blood *104*, 3588-3590.

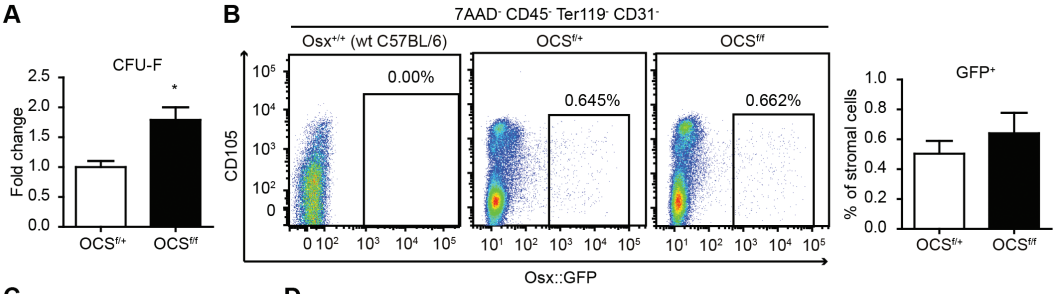
Xiao, Y., Wang, J., Song, H., Zou, P., Zhou, D., and Liu, L. (2013). CD34+ cells from patients with myelodysplastic syndrome present different p21 dependent premature senescence. Leuk. Res. *37*, 333-340.

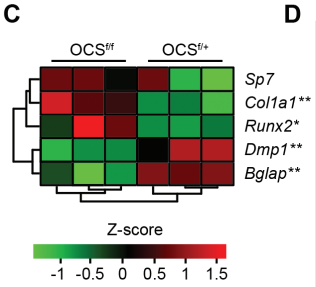
Yahata, T., Takanashi, T., Muguruma, Y., Ibrahim, A.A., Matsuzawa, H., Uno, T., Sheng, Y., Onizuka, M., Ito, M., Kato, S*., et al.* (2011). Accumulation of oxidative DNA damage restricts the self-renewal capacity of human hematopoietic stem cells. Blood *118*, 2941-2950.

Zambetti, N.A., Bindels, E.M., Van Strien, P.M., Valkhof, M.G., Adisty, M.N., Hoogenboezem, R.M., Sanders, M.A., Rommens, J.M., Touw, I.P., and Raaijmakers, M.H. (2015). Deficiency of the ribosome biogenesis gene Sbds in hematopoietic stem and progenitor cells causes neutropenia in mice by attenuating lineage progression in myelocytes. Haematologica *100*, 1285-1293.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL DATA ITEMS





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Gene set	MSigDB	NES	FDR q-val
STRUCTURAL_CONSTITUENT OF RIBOSOME	C5	1.97	0.214
REACTOME_PEPTIDE_CHAIN_ELONGATION	C2	2.38	<0.001
KEGG_RIBOSOME	C2	2.29	<0.001
REACTOME_3_UTR_MEDIATED_TRANSLATIONAL_REGULATION	C2	2.04	0.015
REACTOME_SRP_DEPENDENT_COTRANSLATIONAL_PROTEIN_TARGETING_TO_MEMBRANE	C2	1.98	0.020
REACTOME_TRANSLATION	C2	1.73	0.090
REACTOME_FORMATION_OF_THE_TERNARY_COMPLEX_AND_SUBSEQUENTLY_THE_43S_COMPLEX	C2	1.70	0.102
BILANGES_SERUM_RESPONSE_TRANSLATION	C2	1.64	0.135
LOPEZ_TRANSLATION_VIA_FN1_SIGNALING	C2	1.48	0.239

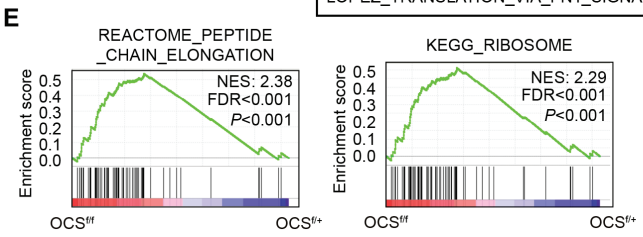
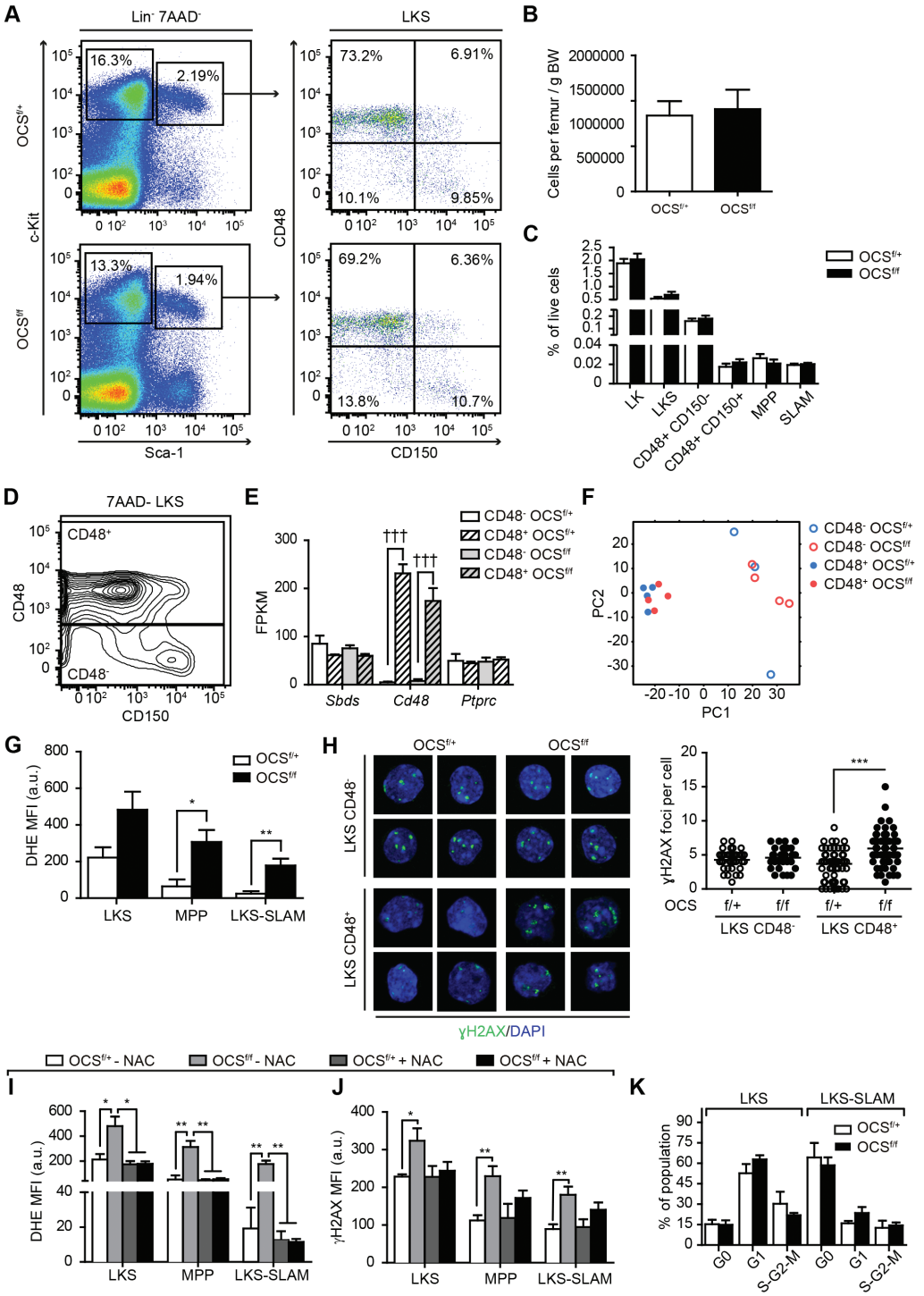


Figure S1. Related to Figure 1; Figure 4. Impairment of terminal osteogenic differentiation in OCS$^{f/f}$ mice. (A) Increased CFU-F numbers (n = 3) with (B) unaltered frequency of Osx::GFP^{+} cells (n = 4) in OCS$^{f/f}$ mice. (C) Depletion of transcripts defining terminal osteogenic differentiation (osteocalcin, *Bglap*) and dentin matrix acidic phosphoprotein 1 (*Dmp1*), critical for proper mineralization of bone, and enrichment of markers of bone progenitor cells or early osteoblasts (*Runx2* and *Col1a1*) in GFP^{+} cells from OCS$^{f/f}$ mice. No statistically significant difference was observed in the expression of osterix (*Sp7*). (D) Significant (FDR<0.25) enrichment of ribosome and peptide chain elongation signatures in OCS$^{f/f}$ GFP^{+} cells (GSEA) with (E) representative plots. *P<0.05. **P<0.01. Data are mean ± SEM.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OCSf/+
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Cells per femur / g BW
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Figure S2. Related to Figure 2. Oxidative and genotoxic stress in HSPCs from OCS$^{f/f}$ mice. (A-C) unaltered HSPC numbers in OCS$^{f/f}$ mice: (A) representative FACS plots, (B) bone marrow cellularity and (C) subset frequency (OCS$^{f/+}$, *n* = 8 in A-B, *n* = 7 in C; OCS$^{f/f}$, *n* = 7). LK: Lin$^-$ c-Kit$^+$ Sca-1$^-$ cells. LKS: Lin$^-$ c-Kit$^+$ Sca-1$^+$ cells. MPP: multipotent progenitors, CD48$^-$ CD150$^-$ LKS cells. SLAM: CD48$^-$ CD150$^+$ LKS cells. (D) FACS isolation of CD48$^+$ and CD48$^-$ HSPC subsets for RNA-seq (OCS$^{f/+}$ CD48$^-$, *n* = 3; other groups, *n* = 4). (E) RNA-seq validation confirming CD48 and *Ptprc* (CD45) expression and the *Sbds*-proficient status of HSPCs from mutant mice. (F) Principal component analysis indicating global preservation of the transcriptome in HSPCs from OCS$^{f/f}$ mice. (G) Superoxide radical accumulation in HSPCs from OCS$^{f/f}$ mice as shown by DHE staining. (H) Increased number of γH2AX foci in HSPCs from mutants (*n* = 2; pooled data). (I-J) NAC treatment attenuates oxidative (I) and (non-significantly) genotoxic stress (J) of OCS$^{f/f}$ mice. NAC- controls were either treated with saline or not injected. (OCS$^{f/+}$ + NAC, *n* = 3; OCS$^{f/f}$ + NAC, *n* = 3;, *n* = 4; other groups, *n* = 5). (K) Ki67 analysis of OCS mice indicating no change in the frequency of G0 HSPCs (OCS$^{f/+}$, *n* = 3; OCS$^{f/f}$, *n* = 4). *P<0.05. **P<0.01. ***P<0.001. $^{+++}$FDR-adjusted P<0.001. Data are mean ± SEM.

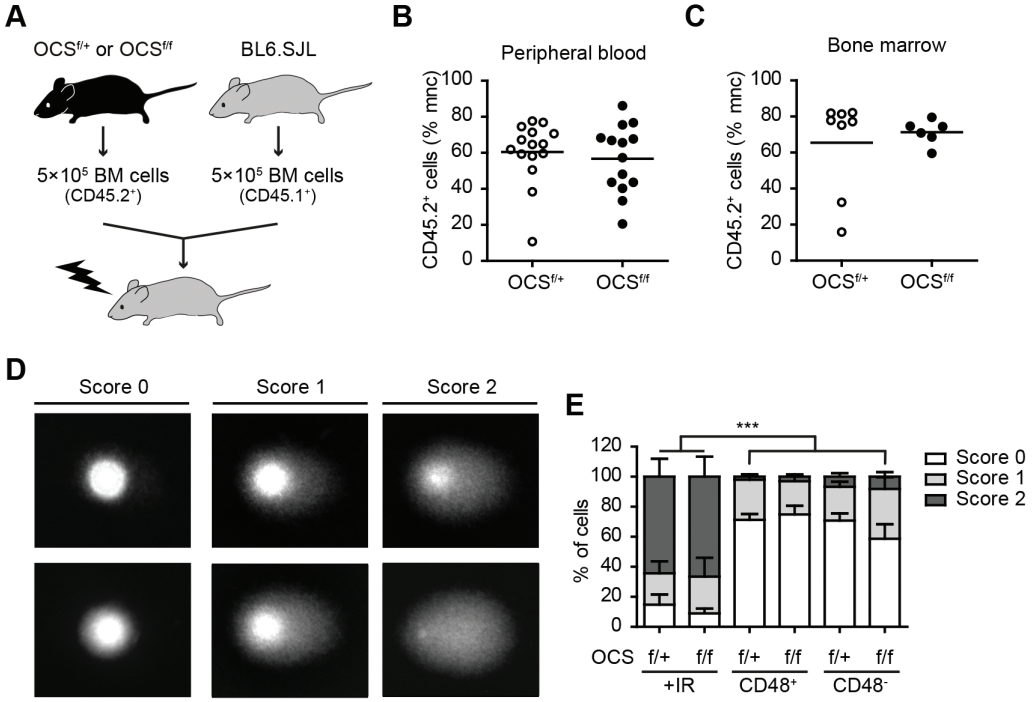

Figure S3. Related to Figure 2. Niche-induced DNA damage does not affect HSPC function. (A) Schematic representation of the competitive transplantation. BM: bone marrow. (B) Peripheral blood frequency of CD45.2$^+$ cells 16 weeks after transplantation. (C) Bone marrow frequency of CD45.2$^+$ cells (21-32 weeks after transplantation). Every circle represents one recipient mouse. (D) Manual scoring system applied to comet assay analysis. (E) Comet assay showing similar frequency of highly damaged HSPCs in OCS$^{f/+}$ and OCS$^{f/f}$ mice (*n* = 5). +IR: 8-10 Gy irradiated positive control (LK cells). ***P<0.001. Data are mean ± SEM.

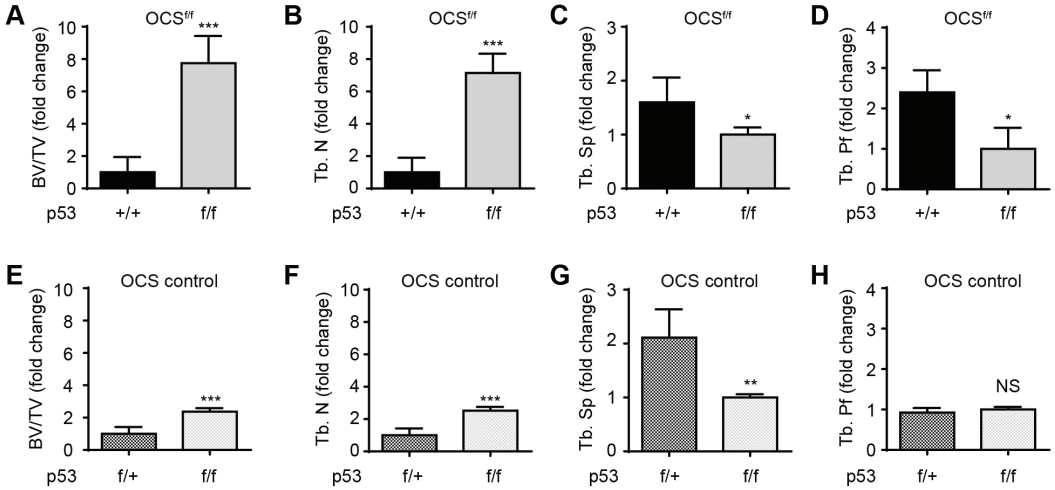

Figure S4. Related to Figure 3. Bone effects of *Trp53* ablation in OCS mutant and control mice. (A-D) Normalization of µCT parameters in OCS$^{f/f}$ p53$^{\Delta}$ mice shown as fold change (p53^{+}, *n* = 3; p53$^{\Delta}$, *n* = 5). (E-F) Modest increase in bone mass upon genetic deletion of p53 in OCS control mice, defined as *Osx*$^{cre/+}$ *Sbds*$^{f/+}$ or *Osx*$^{cre/+}$ *Sbds*$^{+/+}$ (p53$^{f/+}$, *n* = 8; p53$^{f/f}$, *n* = 3). **P*<0.05. ***P*<0.01. ****P*<0.001. Data are mean ± SEM.

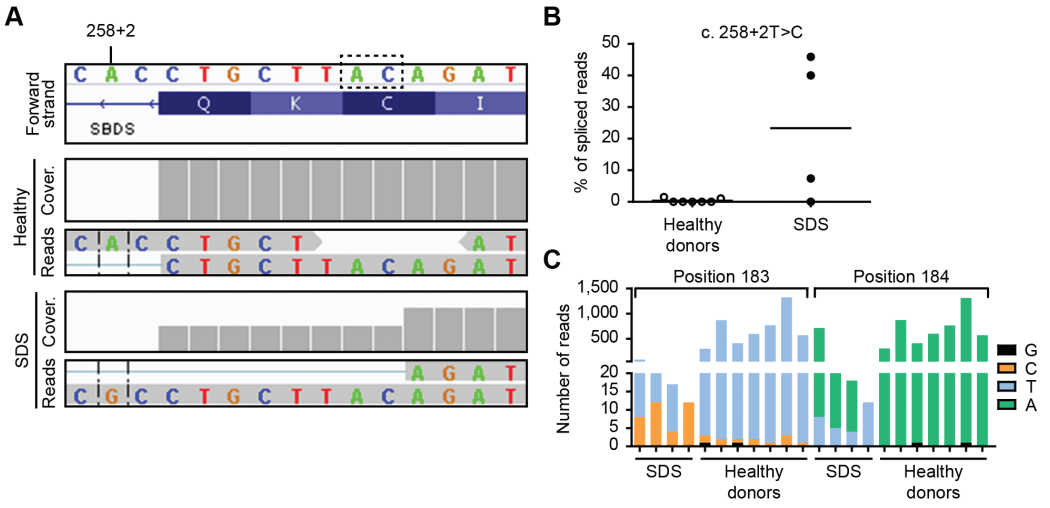

Figure S5. Related to Figure 4. *SBDS* mutations in mesenchymal cells from SDS patients. (A-B) Detection of 258+2 T>C mutation in SDS patients. (A) Representative IGV plot. Note that the coverage level after the cryptic site (dashed box) is reduced in SDS, indicating an 8-bp deletion. (B) Quantification of 258+2 T>C mutation as frequency of spliced reads with 8-bp deletion. Every circle represents a patient. (C) Nucleotide sequence in positions 183-184 from SDS patients and healthy donors.

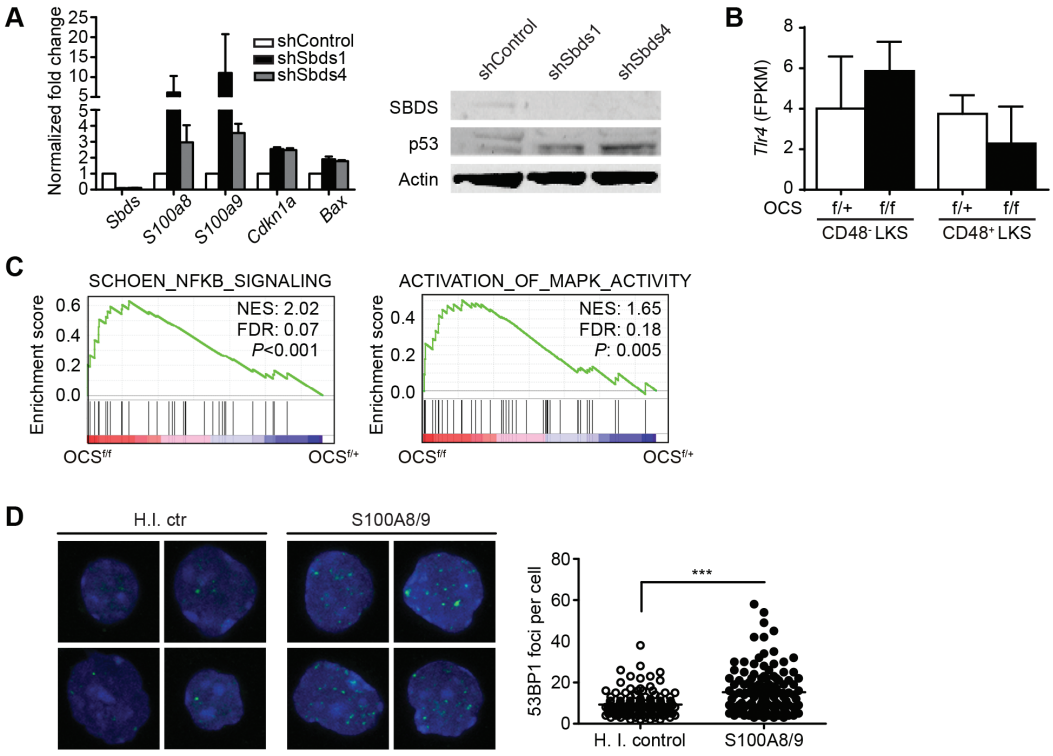

Figure S6. Related to Figure 2; Figure 5. A p53-S100A8/9-TLR axis induces DNA damage in HSPCs. (A) *Sbds*-knock down in OP9 cells induces expression of *S100a8*, *S100a9* and activation of p53 pathway. Left, expression analysis by qPCR (data normalized against shControl, $n = 3$). Right, representative Western Blot analysis showing p53 accumulation upon *Sbds*-knock down. (B, C) TLR4 expression and activation of NF-κB and MAPK pathways in HSPCs from $OCS^{f/f}$ mice (GSEA). NES: normalized enrichment score. (D) *Ex-vivo* treatment with S100A8/9 induces 53BP1 foci of DNA damage in murine HSPCs (LKS cells), Left, representative images. Right, cumulative dot plots ($n = 2$). ***$P < 0.001$. Data in bar graphs are mean ± SEM.

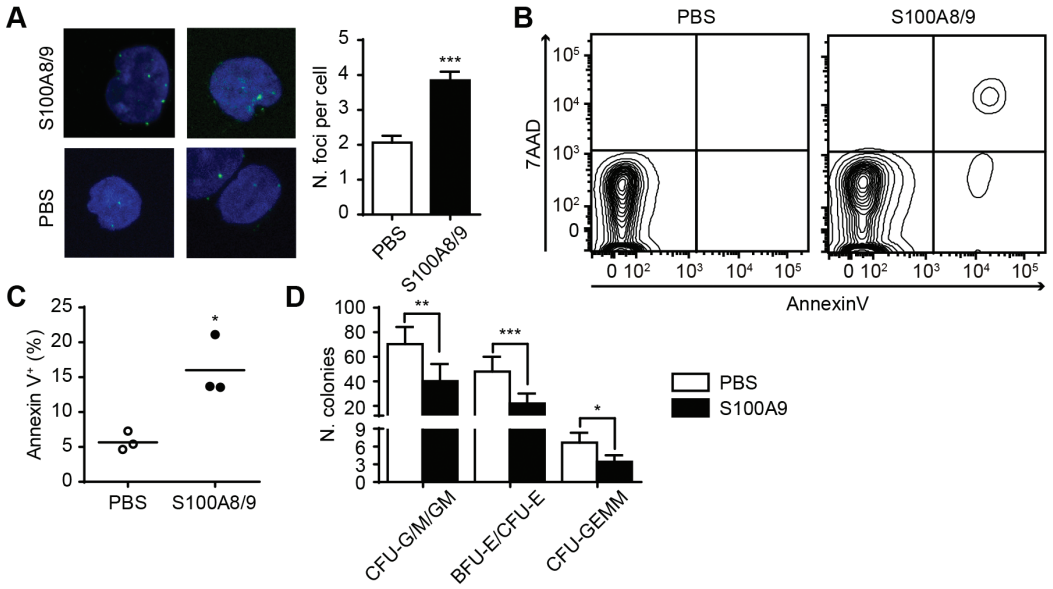

Figure S7. Related to Figure 5. S100A8/9 drives genomic stress in human HSPCs. (A-C) Treatment of human CD34$^+$ HSPCs with recombinant S100A8/9. (A) Induction of γH2AX foci (*n* = 3). (B-C) Increased frequency of apoptotic cells. (B) Representative plot. (C) Quantification (*n* = 3). (D) Reduced colony forming capacity of HSPCs as assessed by CFU-C assay (*n* = 4 independent experiments in triplicate). Data are mean ± SEM. **P*<0.05. ***P*<0.01. ****P*<0.001.

Table S1. Related to Figure 2. Transcriptional profiling of OCS-derived HSPCs reveals dysregulation of signatures previously identified as predictive for leukemia evolution.
Upregulation of G-protein-coupled receptors and cell adhesion-communication, and downregulation of mitochondrial oxidative phosphorylation, ribosome biogenesis, aminoacyl-tRNA synthetase activity, proteasomal degradation, citric acid cycle, cell cycle deregulation and hematopoietic stem cell programs. Normalized enrichment score (NES) and False Discovery Rate-adjusted q-value (FDR) are omitted when FDR > 0.25.

Gene set	MSigDB collection	CD48- LKS		CD48+ LKS	
		NES	FDR	NES	FDR
Enriched in OCS mutants					
G-protein coupled receptors					
G_PROTEIN_COUPLED_RECEPTOR_PROTEIN_SIGNALING_PATHWAY	C5			1.63	0.180
Cell adhesion-communication					
REACTOME_GAP_JUNCTION_ASSEMBLY	C2			1.84	0.170
PID_INTEGRIN_CS_PATHWAY	C2			1.78	0.213
SA_MMP_CYTOKINE_CONNECTION	C2			1.75	0.230
KEGG_CELL_ADHESION_MOLECULES_CAMS	C2			1.70	0.236
Enriched in OCS controls					
Mitochondria and oxidative phosphorylation					
MOOTHA_VOXPHOS	C2	-2.49	<0.001	-1.93	0.001
WONG_MITOCHONDRIA_GENE_MODULE	C2	-2.46	<0.001	-2.19	<0.001
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT_ATP_SYNTHESIS_BY_CHEMIOSMOTIC_COUPLING_AND_HEAT_PRODUCTION_BY_UNCOUPLING_PROTEINS_	C2	-2.44	<0.001	-2.02	<0.001
REACTOME_RESPIRATORY_ELECTRON_TRANSPORT	C2	-2.43	<0.001	-1.91	0.002
KEGG_OXIDATIVE_PHOSPHORYLATION	C2	-2.37	<0.001	-1.75	0.012
REACTOME_MITOCHONDRIAL_PROTEIN_IMPORT	C2	-2.13	<0.001	-1.95	0.001
MOOTHA_MITOCHONDRIA	C2	-2.12	<0.001	-1.96	0.001
HOUSTIS_ROS	C2	-1.58	0.047	-1.34	0.211
REACTOME_RNA_POL_I_RNA_POL_III_AND_MITOCHONDRIAL_TRANSCRIPTION	C2	-1.50	0.076		
GARGALOVIC_RESPONSE_TO_OXIDIZED_PHOSPHOLIPIDS_TURQUOISE_DN	C2	-1.48	0.083		
MOOTHA_HUMAN_MITODB_6_2002	C2			-2.01	<0.001
GALLUZZI_PREVENT_MITOCHONDIAL_PERMEABILIZATION	C2			-1.30	0.248
MITOCHONDRIAL_MEMBRANE_PART	C5	-2.21	<0.001	-1.83	0.016
MITOCHONDRIAL_INNER_MEMBRANE	C5	-2.15	<0.001	-1.99	0.003
MITOCHONDRIAL_PART	C5	-2.13	<0.001	-2.07	0.001
MITOCHONDRIAL_MEMBRANE	C5	-2.07	<0.001	-2.02	0.003
MITOCHONDRIAL_ENVELOPE	C5	-1.98	0.002	-2.01	0.003
MITOCHONDRIAL_MATRIX	C5	-1.97	0.002	-1.81	0.020
MITOCHONDRIAL_LUMEN	C5	-1.96	0.002	-1.81	0.019
MITOCHONDRIAL_RIBOSOME	C5	-1.87	0.008	-1.64	0.060
MITOCHONDRION	C5	-1.85	0.010	-2.01	0.002
MITOCHONDRIAL_RESPIRATORY_CHAIN	C5	-1.81	0.015	-1.87	0.013

Gene set	MSigDB collection	CD48- LKS		CD48+ LKS	
MITOCHONDRION_ORGANIZATION_AND_BIOGENESIS	C5	-1.54	0.121	-1.50	0.133
ELECTRON_CARRIER_ACTIVITY	C5			-1.73	0.034
MITOCHONDRIAL_TRANSPORT	C5			-1.55	0.099
Ribosomes					
BILANGES_SERUM_RESPONSE_TRANSLATION	C2	-1.64	0.028		
REACTOME_DEADENYLATION_DEPENDENT_MRNA_DECAY	C2	-1.63	0.032		
REACTOME_ELONGATION_ARREST_AND_RECOVERY	C2	-1.58	0.046		
REACTOME_TRANSLATION	C2	-2.47	<0.001		
KEGG_RIBOSOME	C2	-2.42	<0.001		
REACTOME_PEPTIDE CHAIN ELONGATION	C2	-2.42	<0.001		
REACTOME_NONSENSE_MEDIATED_DECAY_ENHANCED_BY_THE_EXON_JUNCTION_COMPLEX	C2	-2.37	<0.001		
REACTOME_FORMATION_OF_THE_TERNARY_COMPLEX_AND_SUBSEQUENTLY_THE_43S_COMPLEX	C2	-2.19	<0.001		
MCGOWAN_RSP6_TARGETS_UP	C2			-1.76	0.011
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	C5	-2.33	<0.001		
RIBOSOME	C5	-2.04	0.001	-1.68	0.045
RIBOSOME_BIOGENESIS_AND_ASSEMBLY	C5	-2.02	0.001	-1.78	0.023
ORGANELLAR_RIBOSOME	C5	-1.89	0.007	-1.63	0.067
RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS_AND_ASSEMBLY	C5	-1.83	0.012	-2.11	0.002
RIBOSOMAL_SUBUNIT	C5	-1.78	0.019	-1.62	0.071
TRANSLATION	C5	-1.56	0.118		
PROTEIN_POLYMERIZATION	C5	-1.53	0.126		
RIBONUCLEOPROTEIN_COMPLEX	C5			-2.09	0.001
PROTEIN_RNA_COMPLEX_ASSEMBLY	C5			-1.98	0.003
TRANSLATIONAL_INITIATION	C5			-1.73	0.034
TRANSLATION_REGULATOR_ACTIVITY	C5			-1.71	0.039
TRANSLATION_FACTOR_ACTIVITY_NUCLEIC_ACID_BINDING	C5			-1.70	0.044
TRANSLATION_INITIATION_FACTOR_ACTIVITY	C5			-1.67	0.048
REGULATION_OF_TRANSLATIONAL_INITIATION	C5			-1.55	0.100
tRNA biosynthesis					
REACTOME_CYTOSOLIC_TRNA_AMINOACYLATION	C2			-1.85	0.004
REACTOME_TRNA_AMINOACYLATION	C2			-1.72	0.015
KEGG_AMINOACYL_TRNA_BIOSYNTHESIS	C2			-1.64	0.030
TRNA_METABOLIC_PROCESS	C5			-1.69	0.044
Proteasomal pathway					
KEGG_PROTEASOME	C2	-2.18	<0.001	-1.99	0.001
REACTOME_AUTODEGRADATION_OF_THE_E3_UBIQUITIN_LIGASE_COP1	C2	-2.07	<0.001	-2.01	<0.001
BIOCARTA_PROTEASOME_PATHWAY	C2	-1.92	0.002	-2.14	<0.001
WONG_PROTEASOME_GENE_MODULE	C2			-1.83	0.005

Gene set	MSigDB collection	CD48- LKS		CD48+ LKS	
REACTOME_ANTIGEN_PROCESSING_UBIQUITINATION_PROTEASOME_DEGRADATION	C2			-1.42	0.138
PROTEASOME_COMPLEX	C5	-1.47	0.168	-1.52	0.113
Cell cycle regulation					
REACTOME_SYNTHESIS_OF_DNA	C2	-2.44	<0.001	-2.25	<0.001
REACTOME_S_PHASE	C2	-2.42	<0.001	-2.31	<0.001
CHICAS_RB1_TARGETS_LOW_SERUM	C2	-2.28	<0.001		
ISHIDA_E2F_TARGETS	C2	-2.27	<0.001	-2.11	<0.001
REACTOME_MITOTIC_G1_G1_S_PHASES	C2	-2.27	<0.001	-2.37	<0.001
REACTOME_G1_S_TRANSITION	C2	-2.26	<0.001	-2.31	<0.001
REACTOME_M_G1_TRANSITION	C2	-2.26	<0.001	-2.17	<0.001
REACTOME_DNA_STRAND_ELONGATION	C2	-2.23	<0.001	-2.05	<0.001
KEGG_DNA_REPLICATION	C2	-2.21	<0.001	-2.02	<0.001
RAHMAN_TP53_TARGETS_PHOSPHORYLATED	C2	-2.18	<0.001	-1.90	0.002
REACTOME_CDK_MEDIATED_PHOSPHORYLATION_AND_REMOVAL_OF_CDC6	C2	-2.17	<0.001	-2.00	0.001
REACTOME_ASSEMBLY_OF_THE_PRE_REPLICATIVE_COMPLEX	C2	-2.15	<0.001	-2.20	<0.001
KONG_E2F3_TARGETS	C2	-2.14	<0.001	-2.19	<0.001
REACTOME_DNA_REPLICATION	C2	-2.14	<0.001	-2.29	<0.001
REACTOME_CDT1_ASSOCIATION_WITH_THE_CDC6_ORC_ORIGIN_COMPLEX	C2	-2.13	<0.001	-2.10	<0.001
REACTOME_CYCLIN_E_ASSOCIATED_EVENTS_DURING_G1_S_TRANSITION_	C2	-2.13	<0.001	-2.13	<0.001
MARKEY_RB1_ACUTE_LOF_DN	C2	-2.11	<0.001	-2.27	<0.001
REACTOME_CELL_CYCLE_CHECKPOINTS	C2	-2.10	<0.001	-2.11	<0.001
WHITFIELD_CELL_CYCLE_LITERATURE	C2	-2.08	<0.001	-2.25	<0.001
EGUCHI_CELL_CYCLE_RB1_TARGETS	C2	-2.03	<0.001	-1.97	0.001
REACTOME_REGULATION_OF_MITOTIC_CELL_CYCLE	C2	-2.03	<0.001		
REACTOME_CELL_CYCLE_MITOTIC	C2	-2.02	<0.001	-2.34	<0.001
REACTOME_LAGGING_STRAND_SYNTHESIS	C2	-2.02	<0.001	-1.88	0.003
REACTOME_CELL_CYCLE	C2	-2.01	<0.001	-2.18	<0.001
REACTOME_MITOTIC_M_M_G1_PHASES	C2	-2.00	<0.001	-2.23	<0.001
SCIAN_CELL_CYCLE_TARGETS_OF_TP53_AND_TP73_DN	C2	-1.98	0.001	-1.81	0.006
ZHOU_CELL_CYCLE_GENES_IN_IR_RESPONSE_24HR	C2	-1.90	0.002	-2.12	<0.001
KAUFFMANN_DNA_REPLICATION_GENES	C2	-1.87	0.003	-1.96	0.001
IGLESIAS_E2F_TARGETS_UP	C2	-1.86	0.003	-1.32	0.235
CHANG_CYCLING_GENES	C2	-1.85	0.004	-2.10	<0.001
TANG_SENESCENCE_TP53_TARGETS_DN	C2	-1.82	0.006	-1.86	0.003
MARKEY_RB1_CHRONIC_LOF_UP	C2	-1.78	0.008	-1.42	0.134
REACTOME_G2_M_CHECKPOINTS	C2	-1.78	0.008	-1.71	0.017
ZHOU_CELL_CYCLE_GENES_IN_IR_RESPONSE_6HR	C2	-1.77	0.009	-2.19	<0.001
REACTOME_CYCLIN_A_B1_ASSOCIATED_EVENTS_DURING_G2_M_TRANSITION	C2	-1.68	0.022	-1.39	0.160

Gene set	MSigDB collection	CD48- LKS		CD48+ LKS	
REACTOME_E2F_MEDIATED_REGULATION_OF_DNA_REPLICATION	C2	-1.64	0.028	-1.88	0.003
MOLENAAR_TARGETS_OF_CCND1_AND_CDK4_DN	C2	-1.62	0.033	-2.32	<0.001
REACTOME_G1_S_SPECIFIC_TRANSCRIPTION	C2	-1.62	0.034	-1.94	0.001
BIOCARTA_P53_PATHWAY	C2	-1.59	0.044	-1.31	0.246
VERNELL_RETINOBLASTOMA_PATHWAY_UP	C2	-1.58	0.045	-1.96	0.001
REACTOME_MEIOTIC_RECOMBINATION	C2	-1.57	0.049		
KANNAN_TP53_TARGETS_DN	C2	-1.56	0.052		
IWANAGA_E2F1_TARGETS_INDUCED_BY_SERUM	C2	-1.54	0.059	-1.40	0.155
KEGG_CELL_CYCLE	C2	-1.51	0.068	-1.88	0.003
BIOCARTA_MCM_PATHWAY	C2	-1.49	0.079	-1.54	0.068
REACTOME_G0_AND_EARLY_G1	C2	-1.48	0.086	-1.86	0.003
PID_E2F_PATHWAY	C2	-1.47	0.088	-1.85	0.004
MARKEY_RB1_CHRONIC_LOF_DN	C2	-1.44	0.110		
CHICAS_RB1_TARGETS_GROWING	C2	-1.42	0.121	-1.72	0.015
REACTOME_MITOTIC_PROMETAPHASE	C2	-1.38	0.153	-1.84	0.004
REACTOME_G1_PHASE	C2	-1.37	0.155	-1.65	0.030
BIOCARTA_CELLCYCLE_PATHWAY	C2	-1.35	0.173	-1.65	0.029
HERNANDEZ_MITOTIC_ARREST_BY_DOCETAXEL_1_DN	C2	-1.30	0.220		
WHITFIELD_CELL_CYCLE_G2	C2	-1.30	0.224	-1.60	0.044
SANCHEZ_MDM2_TARGETS	C2	-1.28	0.249		
BENPORATH_PROLIFERATION	C2			-2.15	<0.001
REACTOME_SCFSKP2_MEDIATED_DEGRADATION_OF_P27_P21	C2			-2.14	<0.001
REACTOME_APC_C_CDC20_MEDIATED_DEGRADATION_OF_MITOTIC_ PROTEINS	C2			-2.03	<0.001
REN_BOUND_BY_E2F	C2			-1.89	0.002
REACTOME_PROCESSIVE_SYNTHESIS_ON_THE_LAGGING_STRAND	C2	-1.92	0.001	-1.72	0.016
REACTOME_MITOTIC_G2_G2_M_PHASES	C2			-1.69	0.021
REACTOME_ACTIVATION_OF_THE_PRE_REPLICATIVE_COMPLEX	C2	-1.93	0.001	-1.68	0.022
CEBALLOS_TARGETS_OF_TP53_AND_MYC_UP	C2			-1.67	0.025
REACTOME_RECRUITMENT_OF_MITOTIC_CENTROSOME_PROTEINS_AND_ COMPLEXES	C2			-1.63	0.033
REICHERT_MITOSIS_LIN9_TARGETS	C2			-1.61	0.039
PID_P53REGULATIONPATHWAY	C2			-1.59	0.047
BIOCARTA_G1_PATHWAY	C2			-1.55	0.060
SA_G1_AND_S_PHASES	C2			-1.51	0.082
WHITFIELD_CELL_CYCLE_G2_M	C2			-1.48	0.099
WHITFIELD_CELL_CYCLE_M_G1	C2			-1.48	0.099
WHITFIELD_CELL_CYCLE_G1_S	C2			-1.47	0.101
BIOCARTA_G2_PATHWAY	C2			-1.46	0.112
GEORGES_CELL_CYCLE_MIR192_TARGETS	C2			-1.45	0.116

Gene set	MSigDB collection	CD48- LKS		CD48+ LKS	
WHITFIELD_CELL_CYCLE_S	C2			-1.40	0.155
REGULATION_OF_CYCLIN_DEPENDENT_PROTEIN_KINASE_ACTIVITY	C5	-1.99	0.001		
REPLICATION_FORK	C5	-1.85	0.010	-1.45	0.169
DNA_REPLICATION	C5	-1.83	0.012	-1.58	0.088
CELL_CYCLE_PROCESS	C5	-1.74	0.031	-1.51	0.127
INTERPHASE	C5	-1.67	0.057	-1.45	0.171
CELL_CYCLE_PHASE	C5	-1.64	0.070	-1.40	0.209
CELL_CYCLE_GO_0007049	C5	-1.64	0.070	-1.43	0.174
MITOTIC_CELL_CYCLE	C5	-1.63	0.070	-1.51	0.122
DNA_DEPENDENT_DNA_REPLICATION	C5	-1.59	0.097	-1.52	0.113
INTERPHASE_OF_MITOTIC_CELL_CYCLE	C5	-1.58	0.104	-1.50	0.128
M_PHASE	C5	-1.54	0.122	-1.34	0.250
M_PHASE_OF_MITOTIC_CELL_CYCLE	C5	-1.52	0.125	-1.55	0.098
CELL_PROLIFERATION_GO_0008283	C5	-1.46	0.176		
MITOSIS	C5	-1.43	0.197	-1.56	0.096
DNA_METABOLIC_PROCESS	C5			-1.46	0.160
Metabolism					
REACTOME_TCA_CYCLE_AND_RESPIRATORY_ELECTRON_TRANSPORT	C2	-2.19	<0.001	-2.08	<0.001
MOOTHA_TCA	C2	-1.67	0.024	-1.78	0.008
REACTOME_CITRIC_ACID_CYCLE_TCA_CYCLE	C2	-1.61	0.037	-1.87	0.003
KEGG_CITRATE_CYCLE_TCA_CYCLE	C2	-1.55	0.054	-1.79	0.007
REACTOME_GLUCOSE_METABOLISM	C2	-1.33	0.190		
REACTOME_PYRUVATE_METABOLISM_AND_CITRIC_ACID_TCA_CYCLE	C2			-1.71	0.017
Hematopoietic stem cells					
IVANOVA_HEMATOPOIESIS_INTERMEDIATE_PROGENITOR	C2	-1.67	0.023	-2.02	<0.001
BYSTRYKH_HEMATOPOIESIS_STEM_CELL_QTL_CIS	C2	-1.45	0.103		
PARK_HSC_AND_MULTIPOTENT_PROGENITORS	C2	-1.39	0.145	-1.53	0.070
BYSTRYKH_HEMATOPOIESIS_STEM_CELL_AND_BRAIN_QTL_CIS	C2			-1.32	0.227

Table S2. Related to Figure 2. Stress and DNA damage dysregulation in the transcriptome of HSPCs from OCS$^{f/f}$ mice.

ID	Description	CD48- LKS p-val	CD48+ LKS p-val
GO:0006310	DNA recombination	NS	4.68e-02
GO:0006950	response to stress	4.69e-05	1.31e-10
├GO:0080134	regulation of response to stress	2.25e-03	9.78e-04
└GO:0033554	cellular response to stress	5.53e-06	9.15e-05
├GO:0080135	regulation of cellular response to stress	2.82e-02	NS
└GO:0006974	cellular response to DNA damage stimulus	4.11e-03	1.96e-03
└GO:0006281	DNA repair	3.86e-02	NS
└GO:0006301	postreplication repair	NS	2.78e-02
REAC:5956042	Cell Cycle Checkpoints	1.27e-04	2.86e-04
└REAC:5956049	Activation of ATR in response to replication stress	2.89e-02	4.10e-02
┌REAC:5956279	Gap-filling DNA repair synthesis and ligation in GG-NER	2.01e-02	NS
└REAC:5956420	Gap-filling DNA repair synthesis and ligation in TC-NER	2.01e-02	NS

Genes with significantly different expression (P<0.05) between OCS$^{f/+}$ and OCS$^{f/f}$ mice within the CD48$^-$ or CD48$^+$ populations were interrogated for GO and Reactome term enrichment using g-profiler. P-val: p-value. NS: not significant (P≥0.05).

Table S3. Related to Figure 2. Myc-related signatures depleted in OCS$^{f/f}$ HSPCs.

Gene set	Size	CD48- LKS		CD48+ LKS	
		NES	FDR q-val	NES	FDR q-val
DANG_MYC_TARGETS_UP	140	-2.3691	<0.0001	-2.3219	<0.0001
MENSSEN_MYC_TARGETS	52	-2.2803	<0.0001	-2.1716	<0.0001
YU_MYC_TARGETS_UP	40	-2.1026	<0.0001	-2.2127	<0.0001
ODONNELL_TARGETS_OF_MYC_AND_TFRC_DN	45	-1.9994	0.0004	-1.9883	0.0006
CAIRO_PML_TARGETS_BOUND_BY_MYC_UP	23	-1.9161	0.0015	-1.6278	0.0344
SCHUHMACHER_MYC_TARGETS_UP	80	-1.9014	0.0019	-2.2771	<0.0001
SCHLOSSER_MYC_TARGETS_REPRESSED_BY_SERUM	153	-1.8948	0.0021	-2.1315	0.0001
BENPORATH_MYC_TARGETS_WITH_EBOX	224	-1.8805	0.0025	-1.6005	0.0427
SANSOM_APC_TARGETS_REQUIRE_MYC	195	-1.8271	0.0049	-1.6294	0.0340
PID_MYC_ACTIVPATHWAY	77	-1.7735	0.0088	-1.7577	0.0107
MORI_EMU_MYC_LYMPHOMA_BY_ONSET_TIME_UP	102	-1.7638	0.0098	-1.9423	0.0012
SCHLOSSER_MYC_AND_SERUM_RESPONSE_SYNERGY	32	-1.7246	0.0142	-1.5919	0.0448
KIM_MYC_AMPLIFICATION_TARGETS_UP	192	-1.6787	0.0216	-1.8727	0.0027
DANG_REGULATED_BY_MYC_UP	69	-1.5696	0.0484	-1.7749	0.0091
COLLER_MYC_TARGETS_UP	25	-1.5656	0.0498	-2.0411	0.0003
ACOSTA_PROLIFERATION_INDEPENDENT_MYC_TARGETS_UP	77	-1.4380	0.1081	-1.4098	0.1455
SCHLOSSER_MYC_TARGETS_AND_SERUM_RESPONSE_DN	47	-1.4123	0.1260	-1.9374	0.0012
BILD_MYC_ONCOGENIC_SIGNATURE	193	-1.4101	0.1277	-1.3748	0.1735

Table S4. Related to Figure 4. Human normal donor and patient characteristics.

Sample ID	Disease status	Age	Sex
HD513	Healthy donor	40 y	M
HD723	Healthy donor	48 y	F
HD863	Healthy donor	42 y	M
HD237	Healthy donor	40 y	M
HD703	Healthy donor	39 y	M
HD066	Healthy donor	35 y	M
HD167	Healthy donor	48 y	M
SDS438	SDS patient[a]	9 y	M
SDS132	SDS patient[a]	14 y	M
SDS640	SDS patient[a]	4 y	M
SDS221	SDS patient[a]	18 y	M
MDS247	MDS patient[b]	59 y	M
MDS006	MDS patient[b]	62 y	F
MDS020	MDS patient[b]	71 y	F
MDS159	MDS patient[b]	74 y	F
MDS209	MDS patient[b]	78 y	F
MDS222	MDS patient[b]	66 y	F
MDS610	MDS patient[b]	64 y	F
MDS627	MDS patient[b]	80 y	M
MDS008	MDS patient[b]	67 y	F
DBA044	DBA patient	1 y	F
DBA087	DBA patient	4 mo	M
DBA563	DBA patient	4 y	F

[a]All SDS patients were genetically characterized by compound heterozygosity c.183_184TA>CT/c.258+2T>C. Patients did not receive G-CSF treatment and were not diagnosed with MDS or AML at the time of bone marrow sampling. All patients presented with pancreatic insufficiency (serum trypsinogen level <6 µg/L) and growth retardation (≤ 2 SD). All patients but SDS438 were neutropenic at sampling (absolute neutrophil counts, ANC < 1.5×10^9/l); SDS438 had ANC = 1.62×10^9/l).

[b]See Table S6 for further patient characteristics.

Table S5. Related to Figure S5. In vitro exposure of HSPCs to S100A8/9 activates transcriptional signatures related to TLR signaling and cellular stress/apoptosis.

Toll-like receptor signaling			
BIOCARTA_TOLL_PATHWAY	1.60	0.014	0.404
REACTOME_TOLL_RECEPTOR_CASCADES	1.53	0.005	0.365
PID_TOLL_ENDOGENOUS_PATHWAY	1.49	0.043	0.380
KEGG_TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.46	0.012	0.428
REACTOME_ACTIVATED_TLR4_SIGNALING	1.44	0.025	0.444
REACTOME_INNATE_IMMUNE_SYSTEM	1.56	0.0001	0.374
Activation of p53 and apoptosis pathways			
REACTOME_P53_DEPENDENT_G1_DNA_DAMAGE_RESPONSE	1.52	0.017	0.364
INGA_TP53_TARGETS	1.62	0.019	0.363
RASHI_RESPONSE_TO_IONIZING_RADIATION_1	1.68	0.005	0.326
DAZARD_UV_RESPONSE_CLUSTER_G2	1.69	0.006	0.314
AMUNDSON_GAMMA_RADIATION_RESISTANCE	1.71	0.009	0.316
KEGG_APOPTOSIS	1.74	0.001	0.260
KEGG_P53_SIGNALING_PATHWAY	1.78	0.001	0.217

Table S6. Related to Figure 7. MDS patient characteristics. Provided as an Excel file.

Target	Allele	Primer ID	Sequence	Amplicon size, bp
Sbds	Wild type	a b	CCAGGGTCACGTTAATACAAACC TGAGTTTCAATCCTCAGCATCC	329
	Floxed	a b	CCAGGGTCACGTTAATACAAACC TGAGTTTCAATCCTCAGCATCC	450
	Recombined	c d	TAAAACAAAGCTGCGGTCAAGA ATCCTCAGCATCCCGAACAA	319
Osx	Wild type	e f	CTCTTCATGAGGAGGACCCT GCCAGGCAGGTGCCTGGACAT	No band
	Cre	e f	CTCTTCATGAGGAGGACCCT GCCAGGCAGGTGCCTGGACAT	500
Trp53	Wild type	10.1 10.2	GTTAAGGGGTATGAGGGACA GAAGACAGAAAAGGGGAGGG	400
	Floxed	10.1 10.2	GTTAAGGGGTATGAGGGACA GAAGACAGAAAAGGGGAGGG	600
	Recombined	1.1 10.2	CACAAAAACAGGTTAAACCCAG GAAGACAGAAAAGGGGAGGG	612

All patients were treated with lenalidomide in the context of an ongoing prospective clinical trial (details in Experimental Procedures). Patient characteristics at study entry are listed. Progression free survival is calculated from date of study-entry. Hb, hemoglobin. PLT, platelets. WBC, white blood cells. ANC, absolute neutrophil count. *S100A8* and *S100A9* expression is obtained by RNA-sequencing data. P-values were calculated by Mann-Whitney (MW) test.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mice genotyping and sample collection

DNA was extracted from mouse toes with DirectPCR Lysis Reagent (Viagen Biotech). Genotyping and Cre-mediated recombination were verified on genomic DNA samples using the primers listed in the table below. Mouse bone marrow and bone fraction cells were isolated as previously described (Raaijmakers et al., 2010). Red blood cells (RBC) were lysed with ACK lysing buffer (Lonza) before surface markers staining. Peripheral blood was collected from the submandibular vein in K_2EDTA-coated microtainers (BD) and analyzed using a Vet ABC counter (Scil Animal Care).

Genotyping primers used in the study.

Target	Allele	Primer ID	Sequence	Amplicon size, bp
Sbds	Wild type	a b	CCAGGGTCACGTTAATACAAACC TGAGTTTCAATCCTCAGCATCC	329
	Floxed	a b	CCAGGGTCACGTTAATACAAACC TGAGTTTCAATCCTCAGCATCC	450
	Recombined	c d	TAAAACAAAGCTGCGGTCAAGA ATCCTCAGCATCCCGAACAA	319
Osx	Wild type	e f	CTCTTCATGAGGAGGACCCT GCCAGGCAGGTGCCTGGACAT	No band
	Cre	e f	CTCTTCATGAGGAGGACCCT GCCAGGCAGGTGCCTGGACAT	500
Trp53	Wild type	10.1 10.2	GTTAAGGGGTATGAGGGACA GAAGACAGAAAAGGGGAGGG	400
	Floxed	10.1 10.2	GTTAAGGGGTATGAGGGACA GAAGACAGAAAAGGGGAGGG	600
	Recombined	1.1 10.2	CACAAAAACAGGTTAAACCCAG GAAGACAGAAAAGGGGAGGG	612

Bone mineral density and 3-point bending test analysis

Cortical BMD was calculated from μCT cortical data on the basis of a calibration scanning obtained using two phantoms with known density (0.25 g/cm^3 and 0.75 g/cm^3; Bruker MicroCT) under identical conditions as for the femurs. For the bending test, femurs were placed in a custom-modified Single Column Lloyd LRX System bending device (Lloyd Instruments) and analyzed as previously reported (van der Eerden et al., 2013) using CtAnalyzer software (Bruker MicroCT).

Goldner's Masson trichrome staining

Femurs were embedded in methylmetacrylate as indicated before (Derkx et al., 1998). Sections of 6 µm were deacrylated, hydrated and stained accordingly to the previously described protocol (Gruber, 1992). Images of the metaphysial area were captured with a Nikon Eclipse E400 system (Nikon) using a 20X objective lens. Data was analyzed using Image J software (http://imagej.nih.gov/ij/). Briefly, the bone surface was manually selected and the perimeter length calculated. Osteoblasts were manually identified based on staining and morphology. The frequency of osteoblasts was calculated as percentage of osteoblast area in the bone surface and as number of osteoblasts per mm of bone.

CFU-F assay

Primary bone fraction cells were resuspended in growth medium, consistent of αMEM, 20% FBS (Life Technologies), and Penicillin-Streptomycin solution (Life Technologies), and cultured under hypoxic conditions (5% O_2, 5% CO_2) in 24-well plates (seeding density: 6.5 x 10^4 cells/cm^2). After 24h, the medium was changed to eliminate non-adherent cells. Colonies were stained after 7 days of culture. Briefly, medium was removed and cells were fixed 5' in methanol, stained in a 1:20 dilution of Giemsa staining (Merck Millipore) in distilled water and rinsed with tap water. Colonies were counted with an Olympus CK2 inverted microscope, using a 10X magnification.

Primary cell isolation and flow cytometry

All FACS antibodies incubations were performed in PBS+0.5%FCS for 20 min on ice. To identify hematopoietic stem and progenitor cells (HSPCs), bone marrow cells were first co-stained with a cocktail of biotin-labelled antibodies against the following lineage (Lin) markers: Gr1 (RB6-8C5), Mac1 (M1/70), Ter119 (TER-119), CD3e (145-2C11), CD4 (GK1.5), CD8 (53-6.7) and B220 (RA3-6B2) (all from BD Biosciences). After washing, cells were incubated with Pacific Orange-conjugated streptavidin (Life Technologies) and the following antibodies: Pacific Blue anti-Sca1 (D7), FITC or PE anti-CD48 (HM48-1), PE-Cy7 anti-CD150 (TC15-12F12.2) (all from Biolegend), APC anti-c-Kit (2B8, BD Biosciences).

To analyze differentiated cells and define chimerism, we used FITC anti-Gr1 (RB6-8C5), APC anti-Mac1 (M1/70), PE anti-B220 (RA3-6B2), APC-Cy7 anti-CD45.1 (A20), PE-Cy7 anti-CD45.2 (104), all from Biolegend. To identify stromal cells, bone fraction cell suspension was stained with the following antibodies: APC-Cy7 anti-CD45.2 (104), BV510 anti-Ter119 (TER-119), PE-Cy7 anti-CD105 (MJ7/18), PE anti-CD51 (RMV-7), Pacific Blue anti-Sca1 (D7) (all from Biolegend), PE-CF594 anti-CD31 (MEC 13.3, BD Biosciences).

For human mesenchymal cell isolation, bone marrow aspirates were diluted 1:25 with red blood cell lysis solution (NH_4Cl 0.155 M, $KHCO_3$ 0.01 M, EDTA-Na2.2H2O 0.1 M, pH 7.4)

and incubated for 10 min at room temperature. Cells were collected by centrifugation and washed once with PBS+0.5%FBS. For FACS sorting, immunostaining was performed with the same protocol used for murine bone marrow, using PE CD271 (ME20.4) and PE-Cy7 CD45 (HI30) antibodies (Biolegend). $CD271^+$ cells did not contain erythroid cells based on staining with BV421 CD235a (GA-R2, BD Biosciences).

Apoptosis was assayed with FITC Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the recommendation of the manufacturer. Dead cells were excluded based on 7AAD staining.

The content of p53 in stromal cells was analyzed after cell surface antibody staining and cell permeabilization, obtained with Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences), by incubating cells with Alexa Fluor 647 anti-p53 (1C12) diluted in 1X Perm/Wash buffer (BD Biosciences).

γH2AX levels were assessed in cells fixed and permeabilized with Cytofix/Cytoperm Fixation/ Permeabilization Solution Kit (BD Biosciences) by incubating cells with Alexa Fluor 647 anti-γH2AX (N1-431, BD Biosciences) diluted in 1X Perm/Wash buffer (BD Biosciences).

All FACS events were recorded using a BD LSR II Flow Cytometer and analyzed with FlowJo 7.6.5 software (Tree Star). Cells were sorted with a BD FACSAria III.

SBDS mutation analysis

Mutations in *SBDS* were evaluated in the RNA sequencing data of SDS patients and healthy controls using the Integrative Genomics Viewer (IGV) (Robinson et al., 2011). The c.183_184TA>CT mutation was quantified by annotating the number of reads presenting each of the four different nucleotides for both the positions 183 and 184. Because of its intronic position, the c.258+2T>C mutation was assessed by quantifying the usage of the 251-252 cryptic donor site (Boocock et al., 2003). Specifically, we quantified the fraction of spliced reads with an 8-bp deletion (nucleotides 251-258) as an indication of the mutated genotype.

Mitochondrial membrane potential quantification

After staining cells for surface antigens, cells were washed in PBS+0.5%FBS and centrifuged. Cells were resuspended in PBS+0.5%FBS and tetramethylrhodamine methyl ester (TMRM, Life Technologies) was added from a 10 µM stock solution in DMSO to a final non-quenching concentration of 100 nM. After incubation for 20 min at 37°C, cells were washed with PBS+0.5%FBS and analyzed. Cells treated with 1 µM p-trifluoromethoxy carbonyl cyanide phenyl hydrazone (FCCP) were used as positive control for membrane depolarization.

ROS detection

After surface antigen staining, cells washed in PBS+0.5%FBS and centrifuged. A stock solution of 2.5 µg/µl 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H2DCFDA, Life Technologies) in DMSO was diluted with PBS+0.5%FBS to a final concentration of 3 µg/µl. Cells were resuspended with the CM-H2DCFDA solution and stained for 20 min at 37°C, washed in PBS+0.5%FBS and analyzed.

For dihydroethidium (DHE) studies, the compound (Thermo Fisher Scientific) was reconstituted to a 10mM solution in DMSO. After surface antigen staining and washing, cells were resuspended in 500 µl of diluted DHE solution (1:100,000 in HBSS) and incubated at 37°C for 30 min, then washed in PBS+0.5%FBS and analyzed.

Cell cycle analysis

Mice labeled in vivo by BrdU were sacrificed and bone marrow cells were stained for surface antigen. BrdU staining was performed using the FITC BrdU Flow Kit (BD Biosciences) following the manufacturer's instructions.

For Ki67-based cell cycle analysis, cultured or freshly isolated cells were first stained for surface markers and then fixed and permeabilized with Cytofix/Cytoperm Fixation/ Permeabilization Solution Kit (BD Biosciences). After washing, cells were incubated cells with FITC anti-Ki67 (B56, BD Biosciences) diluted in 1X Perm/Wash buffer (BD Biosciences) for 20 min and then washed. Cells were resuspended in PBS+0.5%FBS and 7AAD was added to detect DNA.

Alkaline comet assay

HSPCs were resuspended in 0.7% low melting agarose (Sigma-Aldrich) in PBS at a concentration of 10^5 cells/ml. 50 µl of cell suspension were spread on CometSlides (Trevigen) and the agarose was allowed to solidify for 30 min at 4°C. Slides were incubated for 1 h at 4°C in lysis buffer (1% Triton-X100 freshly added to 2.5M NaCl, 100 mM EDTA, 10 mM Tris/pH10 solution) protected from light and placed in an electrophoresis tray. After 20 min incubation in alkaline solution (200mM NaOH, 1mM EDTA, freshly prepared), unwound DNA was run in the same solution for 30 min at 1 V/cm. After electrophoresis, slides were washed twice in distilled water for 5 min, fixated in 70% ethanol for 5 min and allowed to dry at 37°C. DNA was stained for 5 min in SYBR Gold (Life Technologies), washed in distilled water and allowed to dry in the dark. Images were captured with a Leica DMRXA fluorescent microscope (10X magnification). DNA damage severity was manually quantified according to the score system depicted in Figure S3D.

Bone marrow transplantation

For serial (competitive) transplantation studies, bone marrow cells from OCS and BL6.SJL mice were isolated and RBC-depleted as described above. BL6.SJL cells were mixed in a 1:1 ratio with cells from $OCS^{f/+}$ of $OCS^{f/f}$ mice. 9 week-old B6.SJL mice were lethally irradiated (8.5Gy) and transplanted with a total of 10^6 bone marrow cells.

For transplantation in S100A9Tg mice, donor bone marrow cells were isolated from 12-week old BL6.SJL mice and transplanted into lethally irradiated (8.5Gy) C57BL/6 or S100A9Tg mice (F10 backcross in C57BL76). Each mouse received 2x106 bone marrow cells by tail vein injection and was sacrificed one month after transplantation.

In all transplantation experiment, recipients received antibiotics in the drinking water for 2 weeks after transplantation.

S100A8/9 measurements

To quantify intracellular levels of S100A8/9 proteins, bone fraction cell suspensions were first stained for surface markers and next fixated and permeabilized with Cytofix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences) according to the manufacturer's instructions. Cells were then resuspended in 1X Perm/Wash buffer (BD Biosciences) and incubated for 20 min with polyclonal rabbit antibodies against mouse S100A8 or S100A9 (Vogl et al., 2014). After washing, cells were incubated for 20 min with Pacific Orange-labelled goat anti-rabbit secondary antibody (Life Technologies) diluted in 1X Perm/Wash buffer, washed and resuspended in PBS+0.5%FBS and analyzed by FACS.

To analyze the concentration of S100A8/9 in the plasma, peripheral blood was collected in Microtainer PST tube (BD) and centrifuged to collect the plasma fraction. Samples were stored at -80°C until the moment of analysis. S100A8/9 was quantified by ELISA as previously described (Vogl et al., 2014).

Immunohistochemical staining of low-risk MDS and age-matched controls (biopsies obtained for disease staging from lymphoma patients without evidence of intramedullary localization) were performed on 5 µm bone marrow sections, which were deparaffinized in xylene and hydrated in a graded series of alcohol. Antigen retrieval was achieved by microwave treatment in citrate buffer (10mM pH 6.0) and blocking of the endogenous peroxidases was performed with 3% H_2O_2 in PBS. Sections were blocked using 10% normal human and goat serum (DAKO) in Teng-T solution followed by overnight incubation at 4°C with primary antibody anti-S100A8 and S100A9 (Vogl et al., 2014) diluted 1:500, CD271 (Sigma Aldrich) diluted 1:200, or normal rabbit immunoglobulin (DAKO) diluted accordingly. Immunoreactions were detected using biotinylated secondary antibody (goat anti-rabbit,

1:2000 dilution) with Vectastain ABC Elite Kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma Aldrich). For all stainings, nuclei were counterstained with haematoxylin (Vector Laboratories). Images of the stained tissue were acquired using a Leica DM5500B upright microscope with 40x lenses and LAS-AF image acquisition software.

Sbds-knockdown and expression analysis in OP9 cells

OP9 cells were grown in DMEM+10%FBS+1%PenStrep. *Sbds* RNA interference was achieved by lentiviral transduction. Briefly, short hairpin RNAs against *Sbds* (shSbds1:TRCN0000108586; and shSbds4:TRCN0000316346) and a commercial non-target control (shControl: SHC002 [SHC]), cloned in the pLKO.1 backbones, were selected from the Mission TRC shRNA library (Sigma-Aldrich). Lentiviral shRNAs were produced in HEK293T cells after cotransfection of shControl, shSbds1, or shSbds4 together with the packaging plasmids pSPAX2 and pMDG.2. OP9 cells were infected with lentivirus for 72 hours in the presence of 4 µg/mL polybrene and selected 72hours with 2 µg/mL puromycin.

RNA isolation, conversion to cDNA and qPCR were performed accordingly to previously described methods (Zambetti et al., 2015), using the SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) for conversion to cDNA. For qPCR, expression levels were obtained using the ddCt method using GAPDH as internal control and shControl sample as calibrator. The following primers were used: *Sbds*-Fw: GCGCTTCGAAATCGCCTG; *Sbds*-Rv: TCTGGTCGTCTGTCCCAAATG; *S100a8*-Fw: ATCACCATGCCCTCTACAAGAATG; *S100a8*-Rv: GTCCAATTCTCTGAACAAGTTTTCG; *S100a9*-Fw: AAGCTGCATGAGAACAACCCA; *S100a9*-Rv: CCCAGAACAAAGGCCATTGA; *Cdkn1a*-Fw: CCTGGTGATGTCCGACCTG; *Cdkn1a*-Rv: CCATGAGCGCATCGCAATC; *Bax*-Fw: CCGGCGAATTGGAGATGAACT; *Bax*-Rv: CCAGCCCATGATGGTTCTGAT.

For protein analysis, cells were lysed in Carin lysis buffer (20 mM Tris-HCl pH 8.0, 138 mM NaCl, 10mM EDTA, 100 mM NaF, 1% NP-40, 10% glycerol, 2mM sodium vanadate) supplemented with 0.5 mM DTT and the protease inhibitor SigmaFast (Sigma Aldrich). 32 µg of protein per condition were denatured and separated on a Novex NuPage 4-12% Bis-Tris Gradient gel (Life Technologies) and transferred to Protran BA83 blotting paper (GE Healthcare Sciences). After blocking, membranes were incubated overnight at 4°C with the following primary antibodies: goat polyclonal anti-SBDS (Santa Cruz, S-15, 1:200); rabbit polyclonal anti- p53 (Leica, CM5, 1:2000). Beta-actin was used as a loading control (mouse monoclonal anti-beta-actin, clone AC-15, Sigma-Aldrich, 1:10,000 dilution). After washing, blots were incubated with the following secondary antibody: IRDye 800CW Donkey anti-Goat IgG (H + L), IRDye® 800CW Donkey anti-Rabbit IgG (H + L), and IRDye 800CW Donkey anti-mouse IgG (H + L) (all from Li-COR and diluted 1:10,000). After final washing steps, Western blots were scanned and processed using an Odyssey Infrared Imager (Li-COR Biosciences).

HSPCs in vitro culture, S100A8/A9 exposure and CFU-C assay

LKS and Lin$^-$ c-Kit$^+$ Sca-1$^-$ cells were sorted from wild type C57BL/6 mice and cultured in a serum-free medium, with the following composition: X-Vivo 15 (Lonza), 1% detoxified BSA, 50 µM β-mercaptoethanol (Life Technologies), 1:100 GlutaMAX™ Supplement (Life Technologies), 20 ng/ml recombinant murine SCF (Peprotech), 100 ng/ml recombinant murine Flt3-Ligand (Peprotech), 1:100 penicillin-streptomycin mixture (Life Technologies). The medium was supplemented with recombinant murine S100A8/9, produced with the same methods described earlier (Vogl et al., 2006), at a final, clinically relevant concentration of 25-50 µg/ml, in the range of concentrations measured in the bone marrow supernatants of MDS patients (List, 2014). A heat-inactivated control was obtained by incubating S100A8/9 at 80°C for 30 min; the protein was cooled-down and next added to the HSPC medium with the same concentration and volume as S100A8/9. LKS and Lin$^-$ c-Kit$^+$ Sca-1$^-$ cells were cultured in 96-well plates at a cell density of $2.5x10^4$ cells/well.

For human studies, cryopreserved CD34$^+$ cells were used, which were isolated from cord blood obtained under informed consent by Ficoll gradient and MACS separation (Miltenyi Biotec). Thawed cells were resuspended in StemSpan SFEM II (STEMCELL Technologies) and recombinant human S100A8/9 was added (R&D systems) at a final concentration of 50 µg/ml. For the control medium, an equal volume of vehicle (PBS) was added. Cells were seeded in flat bottom 96 well-plates ($5x10^4$ cells/well).

For both mouse and human studies, cells were harvested at 4h for γH2AX and cell cycle studies and at 24 h for apoptosis assay.

To assess the effect of S100A9 on HSPC function, CD34$^+$ cord blood cells were resuspended in SFEM1 medium (Stemcell Technologies) containing SCF (50 ng/ml) and human recombinant S100A9 (2.5 µg/ml,ProSpec) or PBS control and seeded in 96-well plates ($2x10^4$ cells/well). After one week of preconditioning (37°C, 5% CO_2), cells were pooled. 2,000 cells per condition were resuspended in 400 µl IMDM and transferred to 3.6 ml of MethoCult H84434 (Stemcell Technologies). Cells were plated in triplicate on 1 cm² petri-dishes (1 ml/dish) and incubated at 37°C/5% CO_2. Colonies were counted after 12-14 days.

SUPPLEMENTAL REFERENCES

Derkx, P., Nigg, A.L., Bosman, F.T., Birkenhager-Frenkel, D.H., Houtsmuller, A.B., Pols, H.A., and van Leeuwen, J.P. (1998). Immunolocalization and quantification of noncollagenous bone matrix proteins in methylmethacrylate-embedded adult human bone in combination with histomorphometry. Bone *22*, 367-373.

Gruber, H.E. (1992). Adaptations of Goldner's Masson trichrome stain for the study of undecalcified plastic embedded bone. Biotech Histochem *67*, 30-34.

List, A. (2014). Myeloid-Derived Suppressor Cells & Altered Innate Immunity in MDS Pathogenesis. http://www.mds-foundation.org/wp-content/uploads/manual/ASH2014/6ListMDSF-ASH-253 12-5-14.pdf

Robinson, J.T., Thorvaldsdottir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat Biotechnol *29*, 24-26.

van der Eerden, B.C., Oei, L., Roschger, P., Fratzl-Zelman, N., Hoenderop, J.G., van Schoor, N.M., Pettersson-Kymmer, U., Schreuders-Koedam, M., Uitterlinden, A.G., Hofman, A.*, et al.* (2013). TRPV4 deficiency causes sexual dimorphism in bone metabolism and osteoporotic fracture risk. Bone *57*, 443-454.

Vogl, T., Eisenblatter, M., Voller, T., Zenker, S., Hermann, S., van Lent, P., Faust, A., Geyer, C., Petersen, B., Roebrock, K.*, et al.* (2014). Alarmin S100A8/S100A9 as a biomarker for molecular imaging of local inflammatory activity. Nat Commun *5*, 4593.

Vogl, T., Leukert, N., Barczyk, K., Strupat, K., and Roth, J. (2006). Biophysical characterization of S100A8 and S100A9 in the absence and presence of bivalent cations. Biochim Biophys Acta *1763*, 1298-1306.

3

ACTIVATION OF NF-κB DRIVEN INFLAMMATORY PROGRAMS IN MESENCHYMAL ELEMENTS ATTENUATES HEMATOPOIESIS IN LOW-RISK MYELODYSPLASTIC SYNDROMES

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HIGHLIGHTS

- Activation of NF-κB signaling in mesenchymal cells is common in LR-MDS.
- Activation of NF-κB in mesenchymal cells leads to transcriptional overexpression of inflammatory factors including negative regulators of hematopoiesis.
- Activation of NF-κB attenuates HSPC numbers and function *ex vivo*.

Myelodysplastic syndromes (MDS) are clonal disorders characterized by ineffective hematopoiesis and the propensity for leukemic transformation. Cumulating evidence has challenged the traditional view that MDS is exclusively driven by hematopoietic cell intrinsic factors. Mesenchymal cells in the bone marrow (BM) microenvironment have emerged as key players in disease pathogenesis, as either initiating or contributing factors.[1-4] We have earlier demonstrated that the transcriptional landscape of highly purified mesenchymal elements from human low risk MDS (LR-MDS) is distinct from normal mesenchymal cells and characterized by cellular stress and the upregulation of inflammatory molecules with known inhibitory effects on normal hematopoiesis.[4] Specifically, mesenchymal overexpression of the alarmins S100A8/9 was shown to drive genotoxic stress in hematopoietic stem/progenitor cells (HSPCs) and are related to leukemic evolution in a subset of LR-MDS patients.[3] An important question emerging from these findings is the nature of the upstream drivers of cellular stress and inflammatory programs in LR-MDS mesenchyme. Here, we show that activation of NF-κB in mesenchymal cells is common in LR-MDS, driving transcriptional activation of inflammatory programs and attenuating HSPC function.

We earlier reported on the elucidation of the transcriptome of highly purified mesenchymal cells isolated from LR-MDS patients (n=45) by massive parallel RNA sequencing[3], suggesting inflammation in these mesenchymal elements. In order to identify candidate master regulatory pathways upstream of inflammatory programs in LR-MDS, we performed Gene Set Enrichment Analysis (GSEA) comparing the transcriptomes of these 45 patients to mesenchymal cells purified from healthy controls (n=10).[4] In total, 120 gene signatures were significantly enriched in the LR-MDS mesenchyme, while 8 signatures were enriched in normal mesenchymal cells. Among the signatures upregulated in LR-MDS patients was a remarkable abundance of signatures related to the activation of the Nuclear Factor-kappa B (NF-κB) family of transcription factors (Figure 1A, 1B). To corroborate the notion of activation of this pathway and provide better insight into the heterogeneity within the population, we assessed the expression levels of NF-κB inhibitor *NFKBIA* (also known as *IκB-α*), which forms an autoregulatory loop with activated NF-κB transcription factors and therefore directly reflects activation of NF-κB signaling.[5, 6] Overexpression of *NFKBIA* was found in the majority of patients, suggesting that mesenchymal NF-κB activation is a common feature in LR-MDS (Figure 1C). To confirm functional activation of NF-κB in mesenchymal elements in LR-MDS, we demonstrated increased phosphorylation of p65, a component of the activated NF-κB complex, in intramedullary located CD271$^+$ mesenchymal cells (Figure 1D) as well as in bone-lining CD271$^+$ stromal cells (Figure 1E-F). Moreover, pathway analysis (GSEA) confirmed transcriptional activation of NF-κB signaling in patients with increased *NFKBIA* expression in their mesenchymal niche cells (Figure S1A and S1B). *NFKBIA* expression was significantly correlated with the expression of inflammatory cytokines and negative regulators of hematopoiesis which are bona fide NF-κB downstream targets such as *IL6*,

IL8, and *CCL3* (Figure S1C). No correlation was found between *NFKBIA* expression and expression of *S100A8* or *S100A9* (spearman correlation -0.11 and -0.22; P-adjusted 0.62 and 0.28, respectively). Patients with activated NF-κB signaling (*NFKBIA*$^{+}$) in mesenchymal niche cells had no significant difference in overall or progression free survival in comparison to the *NFKBIA*$^{-}$ subset (Figure S2A and S2B) in this cohort of uniformly treated LR-MDS patients.[4] No significant correlations were found between mutational status and activation of NF-κB signaling in mesenchymal cells (Figure S2C).

While the genes encoding inflammatory factors and negative regulators of hematopoiesis[4] are *bona fide* downstream targets of NF-κB signaling in other experimental settings, we next wanted to provide experimental support for the view that NF-κB activation specifically in mesenchymal precursor cells results in upregulation of these targets. To this end, we designed a strategy of activating NF-κB signaling in mesenchymal progenitor cells by stably overexpressing the constitutively active form of IKK2 (FLAG-IKK2SE), a kinase upstream regulator of NF-κB, via a lentiviral vector (Figure 2A, Figure S3A-C)[7]in OP9 cells. OP9 cells, like CD271^{+} cells[4], express osteolineage commitment markers as well as HSPC regulatory factors and robustly support the expansion of human HSPCs.[8] NF-κB activation in OP9 cells resulted in overexpression of *NFKBIA* (Figure 2C) and canonical NF-κB downstream negative regulators of hematopoiesis, including *Il6*, *Cxcl2* (murine homologue of *IL8*), *Ccl3*, *Inhba, Fth1, Ltf, Ccl5 and Cxcl4* (Figure 2B), recapitulating findings in LR-MDS patients. Similar to results in OP9 cells, activation of NF-κB in human mesenchymal cells (HS5 cell line and expanded bone-marrow-derived primary mesenchymal cells) (Figure S3D) also resulted in upregulation of NF-κB downstream targets including negative regulators of hematopoiesis such as *IL6, IL8, CCL3, S100A9, INHBA*, and *CCL5* (Figure S3E). Together, the data link the transcriptional landscape of inflammatory alterations in mesenchymal cells to activation of NF-κB in LR-MDS.

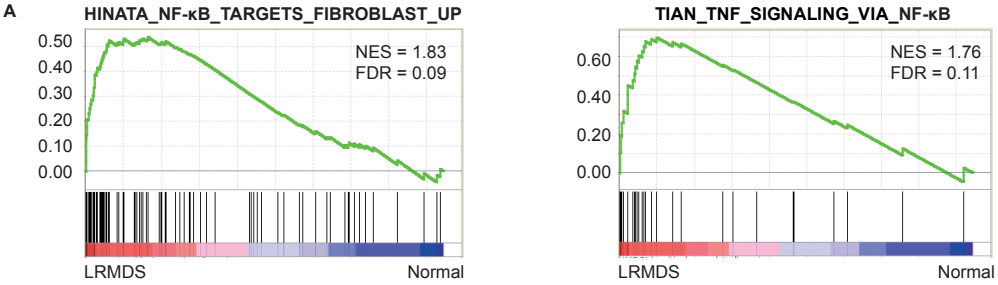
As earlier reported, in human MDS, the majority of CD34^{+} HSPCs is in direct contact with CD271^{+} mesenchymal cells.[9] To assess the effect of NF-κB activation in mesenchymal cells on the biology of normal HSPCs, we performed co-culture experiments with bone marrow CD34^{+} HSPCs and OP9 cells transduced with IKK2SE or empty vector (EV) (Figure 2D). Co-culture for 7 days on IKK2SE-transduced OP9 cells resulted in significantly reduced numbers of immunophenotypically-defined HSPCs in comparison to EV-transduced mesenchymal cells (Figure 2D). In addition, reduced HSPC number was reflected in a reduced number of CFU-Cs (Figure 2E), indicating attenuation of progenitor function in this setting. NF-κB activation resulted in impaired proliferation of mesenchymal cells in serum containing culture conditions (Figure S4A) which may contribute to the reduced support for HSPCs *in vitro* (Figure 2D, left panel). However, equalizing mesenchymal cell numbers by arresting their proliferation (using irradiation and maintenance in non-proliferative, serum free,

culture conditions) (Figure S4B) recapitulated the significantly reduced numbers of immunophenotypically-defined HSPCs on IKK2SE-transduced OP9 cells in comparison to EV-transduced mesenchymal cells (Figure 2D, right panel). This included the fraction enriched for long-term hematopoietic stem cells (LT-HSCs) defined by the markers $CD34^+CD38^-CD45RA^-CD90^+$, multipotent progenitor (MPP; $CD34^+CD38^-CD45RA^-CD90$), multilymphoid progenitor (MLP; $CD34^+CD38^-CD45RA^+CD90^-$), as well as primitive ($CD34^+CD38^-$) and committed $CD34^+CD38^+$ progenitor cells (Figure 2D), indicating that mesenchymal NF-κB signaling attenuates HSPC number and function, at least partially, independent of its effect on mesenchymal cell proliferation.

Collectively, in this brief communication, we demonstrate that mesenchymal NF-κB activation is a common finding in LR-MDS patients leading to transcriptional upregulation of inflammatory programs associated with negative regulation of hematopoiesis and attenuation of HSPC numbers and function.

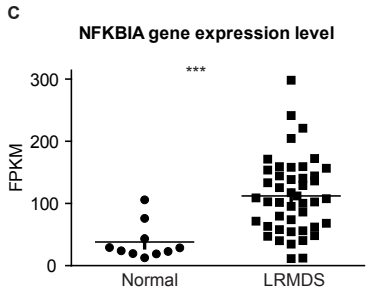
Demonstration of mesenchymal activation of NF-κB provides human disease relevance to a number of murine studies implicating NF-κB activation in ancillary cells to the pathogenesis of hematopoietic disease. NF-κB activation in non-hematopoietic cells has been shown to induce 'MDS-like' myeloproliferative disease (MPD) in mice.[10] In another study, NF-κB activation in bone marrow mesenchymal cells and endothelial cells, as a result of elevated levels of the microRNA miR-155, generated a persistent pro-inflammatory state of the bone marrow niche leading to a MPD-like disease in a Notch/RBPJ loss-of-function mouse model.[11] Our experimental data demonstrate that NF-κB activation in the mesenchyme attenuates normal hematopoiesis, which is of key relevance to LR-MDS characterized by cytopenia. The implication of mesenchymal NF-κB activation in the pathogenesis of MDS may also point towards common mechanisms between the pathogenesis of MDS and oncogenesis in other systems. NF-κB mediated chronic tissue inflammation has been shown to drive cancer initiation and progression via secretion of cytokines and soluble factors in models of several other forms of cancer,[12, 13] including skin, prostate and colon cancer. In these models, activation of NF-κB signaling, specifically in fibroblasts, promoted malignant features in heterotypic (pre)cancerous cells, supporting the hypothesis that mesenchymal inflammation may facilitate tumorigenesis in the hematopoietic system as well.

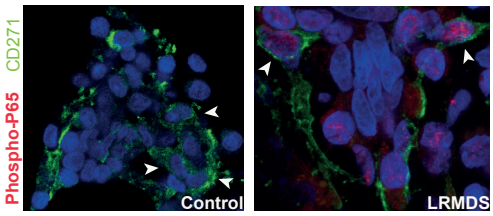
The activation of NF-κB signaling in mesenchymal cells in most LR-MDS patients raises intriguing questions about the events driving this activation. This includes the question whether mesenchymal cell-intrinsic alterations or extrinsic events are driving NF-κB activation. While the answer to this question remains speculative in the absence of experimental evidence (and may vary between patients), it is conceivable that primary alterations in hematopoietic elements drive activation of NF-κB activation in the mesenchymal niche.

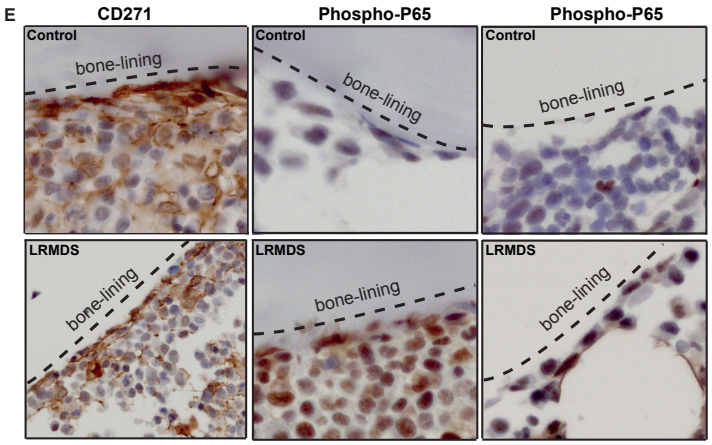


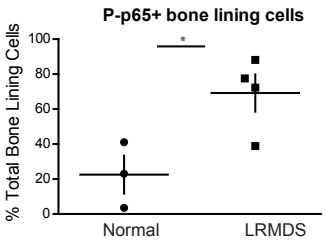
B

Cellular process	Data Sets	Enrichment	Size	NES	FDR q-Val
NF-κB Signaling	HINATA_NF-κB_TARGETS_FIBROBLAST_UP	MDS	84	1.83	0.09
	TIAN_TNF_SIGNALING_VIA_NF-κB	MDS	28	1.76	0.11
	HINATA_NF-κB_TARGETS_KERATINOCYTE_UP	MDS	91	1.76	0.13
	RASHI_NF-κB_TARGETS	MDS	19	1.67	0.15
	HANSON_HRAS_SIGNALING_VIA_NF-κB	MDS	22	1.66	0.16
	SCHOEN_NF-κB_SIGNALING	MDS	34	1.63	0.19









This notion is supported by recent findings where activation of the NF-κB pathway in CD34$^+$ HSPCs is implicated in MDS pathogenesis[14] and CD271$^+$ mesenchymal cells co-localize with CD34$^+$ HSPCs in the bone marrow section of MDS patients.[9] It is therefore reasonable to hypothesize that activated NF-κB pathway in HSPCs signals to the adjacent mesenchymal elements, resulting in NF-κB activation in mesenchymal cells. As NF-κB activation is likely maintained through autocrine/paracrine feedback signaling networks, other cellular types that anatomically localize with the activated HSPCs and mesenchymal elements could be involved as well, suggesting diverse cellular components may participate in this crosstalk. The combined findings suggest that in LR-MDS, activation of NF-κB occurs in both hematopoietic and mesenchymal cells, likely through autocrine and paracrine feedback signaling networks, leading to a NF-κB-mediated inflammatory milieu in the LR-MDS bone marrow and an overexpression of a repertoire of secreted negative hematopoietic regulators. S100A8/9, recently shown by us to induce NF-κB activation and genotoxic stress in HSPCs and to be associated with an increased likelihood of leukemic transformation was not correlated with *NFKBIA* expression in patients, indicating that its regulation is more complex and may include upstream TP53 activation.[3]

Taken together, the findings support the notion that mesenchymal factors, in addition to hematopoietic cell autonomous characteristics, may be therapeutically targeted in LR-MDS and warrant ongoing experiments defining the contribution of NF-κB activation and inflammation to ineffective hematopoiesis and leukemic evolution in MDS.

Figure 1. Activation of NF-κB mediated signaling in LR-MDS mesenchymal cells. (A) Representative GSEA plots demonstrating activation of NF-κB signaling in mesenchymal cells from LR- MDS. (B) Summary of gene sets implicating NF-κB activation in mesenchymal cells from 45 LR-MDS patients. Gene set size, NES, and FDR value of each gene set are as listed. GSEA: gene sets enrichment analysis. NES: normalized enrichment score. FDR: false discovery rate. (C) Gene expression level (in FPKM) of *NFKBIA* in normal and LR-MDS samples. (D) Representative images showing immunofluorescence staining of CD271 and phospho-p65 in both age-matched control (left panel) and LR-MDS patient (right panel) bone marrow slides confirming activation of NF-κB in mesenchymal cells. The white arrow indicates the absence or presence of nuclear phospho-p65 signal (red) in CD271$^+$ (green) mesenchymal cells. The nuclei were counterstained with DAPI. (E) Representative photomicrographs of the distribution of CD271$^+$ mesenchymal cells (left panels). These cells are enriched at the endosteal surface (marked by bone-lining area) and have a spindle-shaped morphology. Representative immuno-histochemical analysis of phospho-p65 (middle and right panels) in age-matched controls (top) and LR-MDS (bottom) patients, demonstrating NF-κB activation in spindle-shaped endosteal cells in LR-MDS. (F) The percentage of phospho-p65+ bone lining cells as a fraction of the total bone lining cells in LR-MDS (n=4) compared to age-matched controls (n=3). *** $P < .001$ * $P < .05$
FPKM: fragments per kilobase of exon per million fragments mapped. *NFKBIA*: NF-Kappa-B Inhibitor Alpha

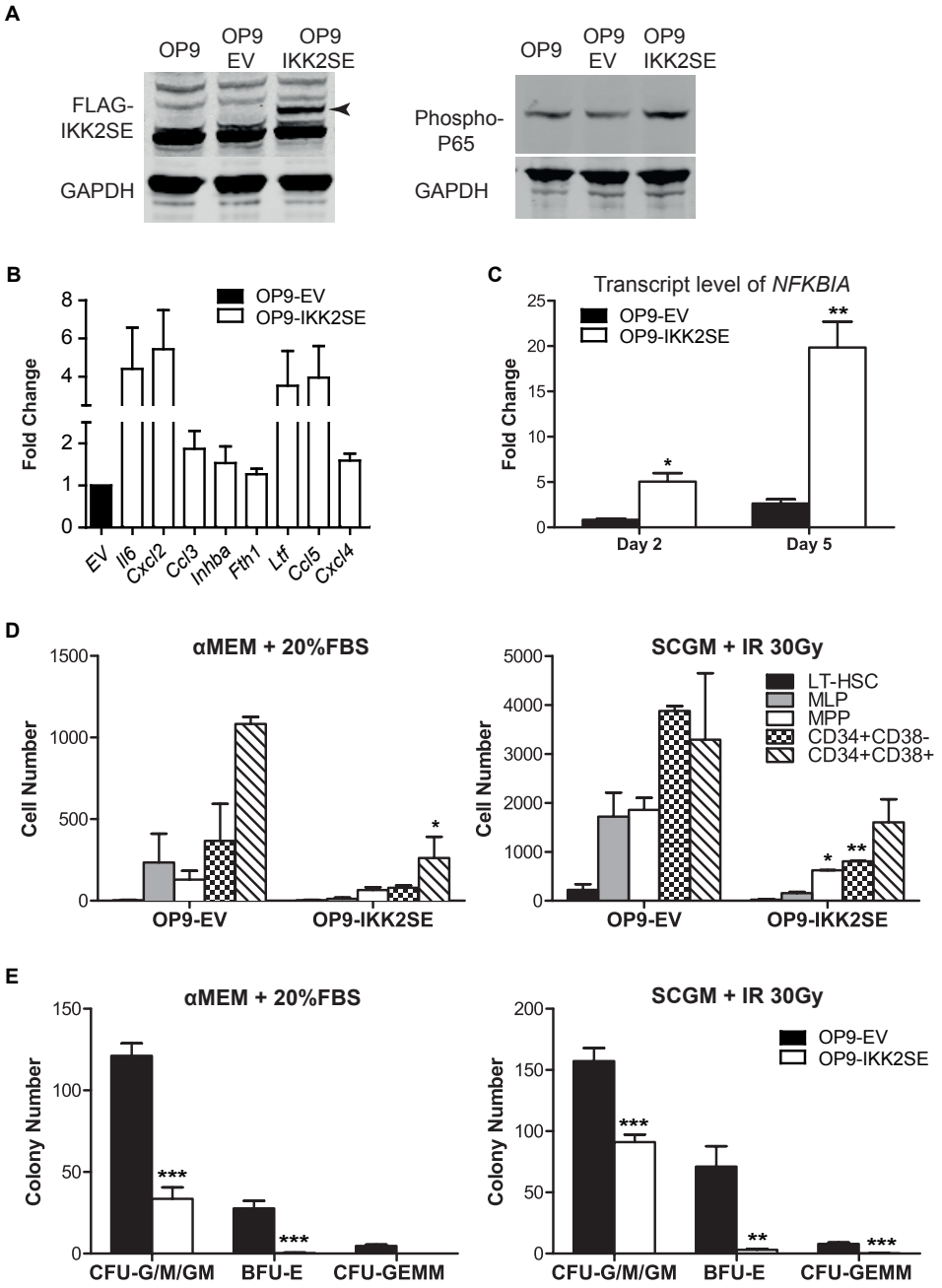

A
OP9
OP9 EV
OP9 IKK2SE
FLAG-IKK2SE
GAPDH
Phospho-P65
B
OP9-EV
OP9-IKK2SE
Fold Change
EV
Il6
Cxcl2
Ccl3
Inhba
Fth1
Ltf
Ccl5
Cxcl4
C
Transcript level of NFKBIA
Day 2
Day 5
D
αMEM + 20%FBS
SCGM + IR 30Gy
Cell Number
LT-HSC
MLP
MPP
CD34+CD38-
CD34+CD38+
E
Colony Number
CFU-G/M/GM
BFU-E
CFU-GEMM

Figure 2. Activated NF-κB signaling in mesenchymal cells attenuates HSPC number and function. (A) Western blot analysis showing the overexpression of Flag-IKK2SE and nuclear phospho-p65, the phosphorylated (active) form of NF-κB, in IKK2SE transduced OP9 cells in comparison to empty vector (EV)-transduced or wild-type cells (B) Expression level of NF-κB downstream targets (*Il6, Cxcl*2) and disease-relevant negative regulators of hematopoiesis[3, 4] in OP9 cells transduced with IKK2SE. Fold change relative to EV is presented. (n=3 for each transcript). (C) Expression level of *NFKBIA* in OP9 cells transduced with EV or IKK2SE and re-plated in serum containing medium for 2 days or 5 days. Fold change relative to wildtype OP9 cells is presented. (n=3). (D) Co-culture experiments with bone marrow $CD34^+$ HSPCs and OP9 stromal cells transduced with either EV or IKK2SE. The co-culture took place for 7 days before analysis. The absolute number of immunophenotypically defined HSPC subsets ($Lin^-CD34^+CD38^+$ progenitors, $Lin^-CD34^+CD38^-$ HSPCs, $Lin^-CD34^+CD38^-CD45RA^-CD90^+$ LT-HSCs, $Lin^-CD34^+CD38^-CD45RA^-CD90^-$ MPPs, $Lin^-CD34^+CD38^-CD45RA^+CD90^-$ MLPs) on day 7 after co-culturing with OP9-EV or OP9-IKK2SE, in serum containing medium condition (left panel) and serum free, Stem Cell Growth Medium (SCGM) condition with OP9-EV and OP9-IKK2SE irradiated at 30 Gy (right panel). Data represent mean +/- SEM of two independent experiments. (E) The total number of CFU-C after 7 days co-culture in serum containing medium condition (left panel) and serum free medium condition with OP9-EV and OP9-IKK2SE irradiated at 30 Gy (right panel). Data represent mean +/- SEM of two independent experiments performed in triplicate. Unpaired t-test was performed for statistical analysis. *** $P < .001$ ** $P < .01$ * $P < .05$

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Author contributions

Z.P, S.C and M.H.G.P.R designed studies; Z.P, S.C, S.J.F.H, K.K, J.F, C.V.D, E.M.J.B, A.M.M and D.J.L-K performed experiments and acquired data; S.C, R.M.H, E.M.J.B, and M.A.S provided technical guidance and bioinformatical analysis; J.N.S provided helpful insights in immunohistochemical data analysis; E.M.P.C and A.vd.L provided patient material; Z.P, S.C and M.H.G.P.R wrote the manuscript; all authors were involved in data interpretation and manuscript reviewing, M.H.G.P.R supervised the study.

Conflict-of-interest disclosure: The authors declare no competing financial interests

REFERENCES

1. Raaijmakers MH, Mukherjee S, Guo S, Zhang S, Kobayashi T, Schoonmaker JA*, et al.* Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* 2010 Apr 8; **464**(7290)**:** 852-857.
2. Medyouf H, Mossner M, Jann JC, Nolte F, Raffel S, Herrmann C*, et al.* Myelodysplastic cells in patients reprogram mesenchymal stromal cells to establish a transplantable stem cell niche disease unit. *Cell Stem Cell* 2014 Jun 5; **14**(6)**:** 824-837.
3. Zambetti NA, Ping Z, Chen S, Kenswil KJ, Mylona MA, Sanders MA*, et al.* Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia. *Cell Stem Cell* 2016 Nov 3; **19**(5)**:** 613-627.
4. Chen S, Zambetti NA, Bindels EM, Kenswill K, Mylona AM, Adisty NM*, et al.* Massive parallel RNA sequencing of highly purified mesenchymal elements in low-risk MDS reveals tissue-context-dependent activation of inflammatory programs. *Leukemia* 2016 Sep; **30**(9)**:** 1938-1942.
5. Brown K, Park S, Kanno T, Franzoso G, Siebenlist U. Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. *Proc Natl Acad Sci U S A* 1993 Mar 15; **90**(6)**:** 2532-2536.
6. Sun SC, Ganchi PA, Ballard DW, Greene WC. Nf-Kappa-B Controls Expression of Inhibitor I-Kappa-B-Alpha - Evidence for an Inducible Autoregulatory Pathway. *Science* 1993 Mar 26; **259**(5103)**:** 1912-1915.
7. Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J*, et al.* IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* 1997 Oct 31; **278**(5339)**:** 860-866.
8. Vodyanik MA, Bork JA, Thomson JA, Slukvin, II. Human embryonic stem cell-derived CD34+ cells: efficient production in the coculture with OP9 stromal cells and analysis of lymphohematopoietic potential. *Blood* 2005 Jan 15; **105**(2)**:** 617-626.
9. Flores-Figueroa E, Varma S, Montgomery K, Greenberg PL, Gratzinger D. Distinctive contact between CD34+ hematopoietic progenitors and CXCL12+ CD271+ mesenchymal stromal cells in benign and myelodysplastic bone marrow. *Lab Invest* 2012 Sep; **92**(9)**:** 1330-1341.
10. Rupec RA, Jundt F, Rebholz B, Eckelt B, Weindl G, Herzinger T*, et al.* Stroma-mediated dysregulation of myelopolesis in mice lackingk l kappa B alpha. *Immunity* 2005 Apr; **22**(4)**:** 479-491.
11. Wang L, Zhang H, Rodriguez S, Cao L, Parish J, Mumaw C*, et al.* Notch-dependent repression of miR-155 in the bone marrow niche regulates hematopoiesis in an NF-kappaB-dependent manner. *Cell Stem Cell* 2014 Jul 03; **15**(1)**:** 51-65.
12. DiDonato JA, Mercurio F, Karin M. NF-kappaB and the link between inflammation and cancer. *Immunol Rev* 2012 Mar; **246**(1)**:** 379-400.
13. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S*, et al.* NF-kappaB functions as a tumour promoter in inflammation-associated cancer. *Nature* 2004 Sep 23; **431**(7007)**:** 461-466.
14. Wei Y, Chen R, Dimicoli S, Bueso-Ramos C, Neuberg D, Pierce S*, et al.* Global H3K4me3 genome mapping reveals alterations of innate immunity signaling and overexpression of JMJD3 in human myelodysplastic syndrome CD34+ cells. *Leukemia* 2013 Nov; **27**(11)**:** 2177-2186.

SUPPLEMENTARY INFORMATION

Supplementary materials and methods

Patient and healthy donor bone marrow samples

Bone marrow samples from LR-MDS patients and healthy donors were obtained as previously described, including the clinical characteristics of the patients and information about the healthy donors.[1, 2] In short, all 45 patients were included in the HOVON89 clinical trial (HOVON89; www.hovon.nl; www.trialregister.nl as NTR1825; EudraCT No. 2008-002195-10) where patients with low or intermediate-1 risk MDS based on IPSS criteria were recruited. The healthy controls were obtained from donors for allogeneic transplantation. All samples were collected with informed consent, approved by the Institutional Review Board of the Erasmus Medical Center, the Netherlands, in accordance with the declaration of Helsinki.

Flow cytometry analysis

Sorting of the mesenchymal cells from the bone marrow of patients and normal donors were performed by FACS as previously described.[1, 2] The cells were directly sorted in 800ml Trizol (Ambion) for RNA isolation.

For immunophenotyping of CD34+ HSPCs after 7 days of co-culture, the following antibodies were used based on earlier work with optimized dilutions: Lin-APC (1:25), CD34-PE-Cy7 (1:30), CD38-PercP-Cy5.5 (1:60), CD90-PE (1:30) and CD45RA-APC-H7 (1:30).[3] Live cells were selected based on the DAPI (1:7500) gate. The gating strategy defining the different subsets of HSPCs is illustrated in supplemental figure 3F-G. The OP9 stromal cells were excluded based on ZsGreen expression, as the transduced OP9 cells were ZsGreen (GFP) positive (Figure S3C). The data was acquired using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software.

Counting of the cells after 7 days of co-culture was accurately obtained with flow-count fluorosphere beads (Beckman Coulter), in combination with the following antibodies: CD34-PE-Cy7 (1:50) and CD45-BV510 (1:50). The gating strategy is shown in Figure S3H. The data was acquired using a LSRII flow cytometer (BD Biosciences) and analyzed using FlowJo software. The number of cells was calculated based on the following formula: CD34+ cells per ml = (Live CD34+ events x bead concentration)/number of acquired single beads/(volume beads/volume cells). The number of total mononuclear cells (MNCs) determined by cell counting with beads and frequency of each HSPCs subpopulation from immunophenotyping were combined to obtain the number of cells present in individual subpopulations.

RNA extraction and RNA quality control

Total sample RNA isolation was performed according to the standard protocol of RNA isolation with Trizol and GenElute LPA (Sigma). In the end, the RNA pellet was resuspended in 7.5ml of RNAse free water (Qiagen) and quality and quantity of the total RNA was checked on a 2100 Bio-analyzer (Agilent) using the Agilent RNA 6000 Pico Kit.

RNA sequencing and gene expression profiling

RNA sequencing and the downstream data processing were performed as previously described.[1, 2] In short, SMARTer Ultra Low RNA Kit (Clonetech) for Illumina Sequencing was used to prepare the cDNA based on the manufacture's protocol. Once the cDNAs were obtained, the subsequent library preparation steps, sequencing and alignments were performed as earlier described, using a range of tools including the cutadapt program, TopHat2 and cufflinks.[4] The resulting gene expression values were measured as FPKM (Fragments per kilobase of exon per million fragments mapped). Fragment counts were determined per gene with HTSeq-count, utilizing the strict intersection option, and subsequently used for differential expression analysis using the DESeq2 package, with standard parameters, in the R environment. Multiple testing correction was performed with the Benjamini-Hochberg procedure to control the False Discovery Rate (FDR). Finally, gene set enrichment analysis (GSEA) was performed on the FPKM values using the curated C2 collection of gene sets within MSigDB.[5]

Direct comparison between gene expression data from purified and *ex vivo* expanded LR-MDS mesenchymal cells was performed as previously described.[2] In brief, BAM files containing the molecular data of *ex vivo* expanded stromal cells derived from LR-MDS patients (n=5)[6] were obtained from the European Genome-Phenome Archive data base (EGAS00001000716). GSEA was then performed comparing our gene expression data of the purified LR-MDS mesenchymal cells (n=45) to the gene expression data of expanded stromal cells (n=5).[5]

Immunohistochemistry and immunofluorescence

Immunohistochemical and immunofluorescence stainings of LR-MDS and control paraffin-embedded bone marrow slides were performed as previously described.[7] CD271 and phospho-p65 antibodies were used to mark the stromal cells of interest with their corresponding phospho-p65 status. Immunohistochemical single staining facilitated the enumeration of phospho-p65$^+$ bone lining stromal cells; whereas the immunofluorescence double staining demonstrated the co-localization of CD271$^+$ and phospho-p65$^+$ cells in patient and control sections.

All the bone marrow sections (5-µm) were deparaffinized in xylene and hydrated in a graded series of alcohol. Antigen retrieval was achieved by microwave treatment in citrate buffer

(10mM pH 6.0) and blocking of the endogenous peroxidases was performed with 3% H_2O_2 in PBS. For IHC staining, sections were blocked using 10% normal human and goat serum (DAKO) in Teng-T solution (10 mM Tris, 5 mM EDTA, 0.15 M SodiumCl, 0.25 % gelatine, 0.05 % Tween-20) followed by an overnight incubation at 4°C with primary antibody: rabbit anti-human phospho Serine 276 of p65 (p-p65, active form; Santa Cruz Biotechnology) diluted 1:400, CD271 (Sigma Aldrich) diluted 1:200, or normal rabbit immunoglobulin (DAKO) diluted accordingly. Immunoreactions were detected using biotinylated secondary antibody (goat anti-rabbit, 1:2000 dilution) with Vectastain ABC Elite Kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride (Sigma Aldrich). For all stainings, nuclei were counterstained with haematoxylin (Vector Laboratories). Images of stained tissues were acquired using a Leica DM5500B upright microscope with 40x lenses and LAS-AF image acquisition software (Leica). The number of phospho-p65+ bone lining cells were manually quantified using ImageJ software from x40 photomicrographs of blindly selected trabecular bone area. Unpaired t-test was performed and $p < .05$ was considered significant.

For IF staining, sections were blocked with 10% normal human, goat and horse serum (DAKO) in Teng-T solution followed by an overnight incubation at 4°C with a primary antibody directed against phospho Serine 276 of p65. The next day, biotinylated goat anti-rabbit secondary antibody and streptavidin-Dylight 594 (1:200, Vector Laboratories) were incubated consecutively. This was followed by an additional incubation with a primary mouse anti-human antibody directed against CD271 (1:100, eBioscience) followed by conjugated horse anti-mouse Dylight 488 (1:200, Vector Laboratories). Counterstain with DAPI and mounting were performed using Vectashield mounting medium (Vector Laboratories, H-1200). Control slides with single stains for the primary antibodies CD271 or phospho-p65 combined with all conjugated secondary antibodies were performed as a background control. Images were acquired on a Leica SP5 confocal laser scan microscope equipped with Diode/Argon/HeNe lasers using a 40x planapochromat oil immersion objective. Images were analyzed using ImageJ software.

Lentiviral transduction of OP9, HS5 and primary expanded stromal cells

The plasmid containing mutant form of IKK-2 (FlagIKK-2 S177E S181E; referred hereafter as IKK2SE), which has previously been demonstrated to result in constitutive NF-κB activation, was obtained from Addgene.[8, 9] The Flag-IKK2SE fragment was recloned into the pHAGE ires ZsGreen lentiviral vector (kindly provided by Dr. R. Delwel). Stable transduction of OP9 cells (ATCC, CRL-2749) with pHAGE-EV and IKK2SE was performed and transduction efficiency was determined by FACS measuring ZsGreen expression of the cells (Figure S3C). Overexpression of IKK2SE was confirmed by Western blotting against Flag-tag and the subsequent activation of NF-κB was verified using an antibody against the active form of NF-κB: phospho-p65 (Ser 536; Cell Signaling).

Lentivirus for both pHage-EV or pHage IKK2SE (Flag-tagged IKK2 S177E S181E) was produced in 293T cells, by means of transient transfection of the pHage EV or pHage IKK2SE, together with the packaging plasmids pSPAX and pMD2.G. OP9 cells were transduced with equal titer lentivirus and 3 days later OP9-EV or OP9-IKK2SE were used for subsequent co-culture experiments.

The same transduction procedures were carried out for human HS5 stromal cell line (ATCC, CRL-11882) and primary expanded stromal cells. All cell lines used in this study were authenticated and mycoplasma free; all cell lines were maintained in cell culture medium, consist of Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplied with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Luciferase Reporter Assay

We performed NF-κB responsive luciferase reporter assays to test the functionality of the recloned pHage IKK2SE construct. HEK293T cells were transiently co-transfected with a luciferase vector containing three NF-κB responsive elements together with either pHage EV or pHage IKK2SE. Cells were harvested after 48 hours and luciferase activity was measured with the Dual-Luciferase Reporter Assay System (Promega) using a Victor3 plate reader (PerkinElmer). As a control for the luciferase assay pMIG EV and pMIG HA-IKK2SE were used (Figure S3A and S3B). Luciferase vector containing 3x NF-κB responsive elements and pMIG HA-IKK2SE were kindly provided by Dr. H. Schepers and J.J. Schuringa.[10]

Western Blotting

Protein extracts were made by lysing cells in Carin lysis buffer (20 mM Tris-HCl pH 8.0, 138 mM NaCl, 10mM EDTA, 100 mM NaF, 1% Nonidet P-40, 10% glycerol, 2mM NA-vanadate) supplemented with 0.5 mM DTT and the protease inhibitor SigmaFast (Sigma Aldrich). Equal amounts of proteins were denatured and separated on a Novex NuPage 4-12% Bis-Tris Gradient gel (Life Technologies) and transferred to Protran BA83 blotting paper (GE Healthcare Life Sciences). Primary antibodies mouse anti-Flag (1:1000) (Sigma Aldrich) or Rabbit anti-phospho p65 S536 (1:1000) (Cell Signaling) were incubated overnight at 4°C after necessary blocking step. Flag antibody was used to confirm transfection or transduction efficiency; whereas phospho-P65 was used to demonstrate phospho-p65 overexpression in IKK2SE transduced cells. As secondary antibodies (1:10000 for both) donkey anti-mouse 800 or goat anti-rabbit 800 (Li-COR Biosciences) were used. GAPDH (Santa Cruz Biotechnologies) was used as a loading control (primary: rat anti-GAPDH (1:1000), secondary: donkey anti-rat 680 (1:10000). After the final incubation, Western blots were scanned and processed using an Odyssey Infrared Imager (Li-COR Biosciences).

Quantitative PCR

Total RNA of OP9-EV, OP9-IKK2SE, HS5-EV, HS5-IKK2SE, primary stroma-EV, primary stroma-IKK2SE cells was isolated 3 days after transduction, or at time points as indicated otherwise, using Trizol reagent (Ambion), followed by cDNA synthesis using SuperScript II Reverse Transcriptase II kit (Invitrogen). Q-PCR was performed using Fast SYBR Green master mix (Applied Biosystems) on a 7500 Fast Real-Time PCR system (Applied Biosystems) with the following primer sets:

Murine: *Il6*: Fw 5'-TCGGAGGCTTAATTACACA-3' Rv 5'-CTGGCTTTGTCTTTCTTGTT-3; *Cxcl2* (murine homologue of IL8): Fw 5'-GCGCCCAGACAGAAGT-3' Rv 5'-CGGGTGCTGTTTGTTTT-3'; *Ccl3*: Fw 5'-TTCTCTGTACCATGACACTCTGC-3' Rv 5'-CGTGGAATCTTCCGGCTGTAG-3'; *Inhba*: Fw 5'-TCACCATCCGTCTATTTCAGCA-3' Rv 5'-CTTCCGAGCATCAACTACTTTCT-3'; *Nfkbia*: Fw 5'-TGAAGGACGAGGAGTACGAGC-3' Rv 5'-TTCGTGGATGATTGCCAAGTG-3'; *Fth1*: Fw 5'-CAAGTGCGCCAGAACTACCA-3' Rv 5'-GCCACATCATCTCGGTCAAAA-3'; *Ltf*: Fw 5'-TGAGGCCCTTGGACTCTGT-3' Rv 5'-ACCCACTTTTCTCATCTCGTTC-3' *Ccl5*: Fw 5'-GCTGCTTTGCCTACCTCTCC-3' Rv 5'-TCGAGTGACAAACACGACTGC-3'; validated all-in-one murine C*xcl4* (PF4) primers (Genecopoeia).

Human*: IL6*: Fw 5'-CCCCCAGGAGAAGATTC-3' Rv 5'- GCTGCTTTCACACATGTTACT-3'; *IL8*: Fw 5'- CCGGAAGGAACCATCT-3' Rv 5'-TTGGGGTGGAAAGGTT-3'; *CCL3*: Fw 5'-GCAACCAGTTCTCTGCATCA-3' Rv 5'-TGGCTGCTCGTCTCAAAGTA-3'; *INHBA*: Fw 5'-ACGGGTATGTGGAGATAGAGGA-3' Rv 5'- GGACTTTTAGGAAGAGCCAGACT-3'; *CCL5*: Fw 5'-CGCTGTCATCCTCATTGCTA-3' Rv 5'-CCATTTCTTCTCTGGGTTGG-3'; *S100A9*: Fw 5'-TTCAAAGAGCTGGTGCGAAAAG-3' Rv 5'-GCATTTGTGTCCAGGTCCTCC-3'; *Ikk2SE*: Fw 5'-GGTGAGCAGATTGCCATCAAG-3' Rv 5'- ACCCTCAGTTCGCTGGTCTCG-3'

The expression level of each molecule was normalized against the housekeeping gene *Hprt* (in OP9) or *GAPDH* (in HS5 or primary expanded stromal cells) and the fold change of the expression level of each gene in IKK2SE-transduced cells was calculated relative to EV transduced cells or to wildtype OP9 cells as indicated.

Isolation and culture of bone marrow CD34+ cells

Human bone marrow $CD34^+$ cells (obtained from healthy donors) were selected by MiniMacs (Miltenyi Biotec) followed by LS and MS MACS separation columns (Miltenyi Biotec) to achieve optimal purity. In the co-culture setting, 38000 OP9-EV or OP9-IKK2SE cells per 1.9cm^2 were plated in cell culture medium one night before the experiment. The next morning, where applicable, irradiation of OP9-EV or OP9-IKK2SE monolayers was performed in RS320 RS X-Ray Cabinet (Xstrahl) at a single dosage of 30 Gray (Gy). Subsequently 15000 $CD34^+$ cells per 1.9cm^2 were resuspended in either serum containing medium or serum free medium, and

directly plated on either a OP9-EV or a OP9-IKK2SE monolayer (Figure 2D). Serum containing culture medium consisted of αMEM (GE Healthcare Life Sciences) supplemented with 20% FBS, Serum free culture medium consisted of GMP serum-free Stem Cell Growth Medium (SCGM, CellGenix); both media were further supplied with 1% penicillin/streptomycin, 50 ng/ml stem cell factor (SCF), 50 ng/ml FLT3 and 50 ng/ml thrombopoietin. Cultures were kept at 37°C in a 5% CO_2 incubator for 7 days until further analysis.

CFU-C

On day 7 of the co-culture, 4000 mononuclear cells (MNCs) from OP9-EV condition, and equal volume of cells in OP9-IKK2SE condition (applicable to both serum containing and serum free conditions) were collected and resuspended in IMDM medium. This cell suspension was added to MethoCult™ GF H84434 (StemCell Technologies), which allows the growth of colonies from all three lineages, and triplicate dishes were plated. The methocult plates were kept at 37°C in a 5% CO_2 incubator for 2 weeks until colony counting.

Correlation plots

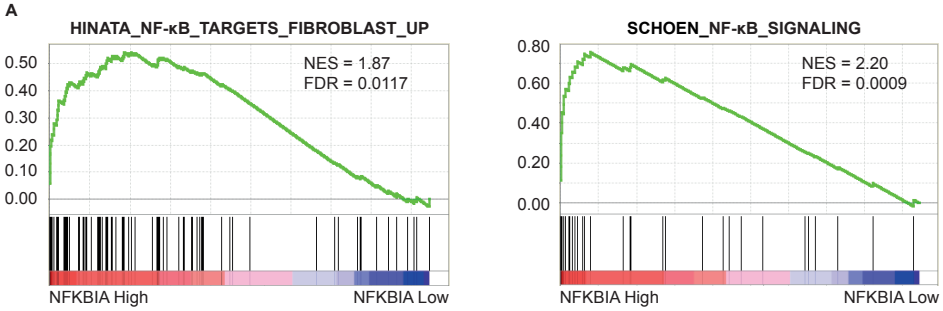
Correlation plots for the specified genes were based on FPKM values, obtained from our previous work. [1,2] Non-parametric correlation estimates (Spearman's rho) and corresponding two-sided p-values were calculated using the cor.test() function available in base R (version 3.4.1) on Windows 10. The FDR was used to adjust P-values for multiple testing. FDR-corrected p-values smaller or equal to 0.05 were considered statistically significant.

Clinical outcome of LR-MDS patients

Overall survival curves were calculated using the survival R package. Alive patients were censored at final follow-up and deaths from any cause were considered events. The proportional hazard assumption was visually inspected using (-log(-log(S(t)))) plots. The Peto & Peto modified Gehan-Wilcoxon test was used to test for statistically significant differences in the distribution of survival times between *NFKBIA* subgroups (P-values ≤ 0.05 were considered statistically significant). To investigate the possible differences in progression-free survival in both *NFKBIA* subgroups, we calculated the cause-specific cumulative incidence probabilities for progression of disease (defined as an increase in blasts, either in bone marrow or in peripheral blood [see Appendix C of the HOVON89 clinical trial protocol], or development of leukaemia), while accounting for competing risks. To this end, we made use of the cuminc() function from the cmprsk R package. We tested for differences in cumulative incidence probabilities with Gray's test, implemented in the cuminc() function of the cmprsk R package. Visual representations of the data were obtained with the help of the ggplot2, ggfortify and survminer R packages for survival data.

Supplementary Figures

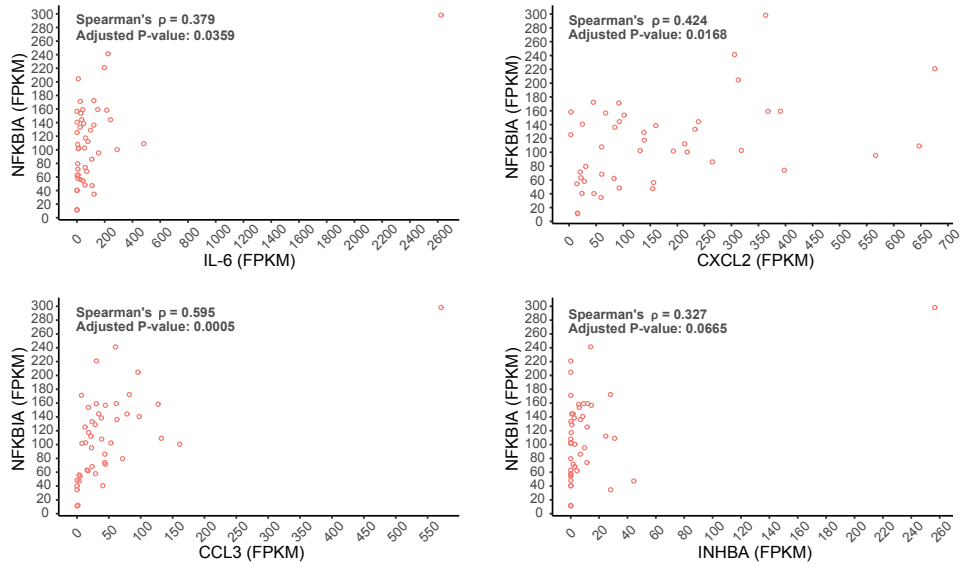
A



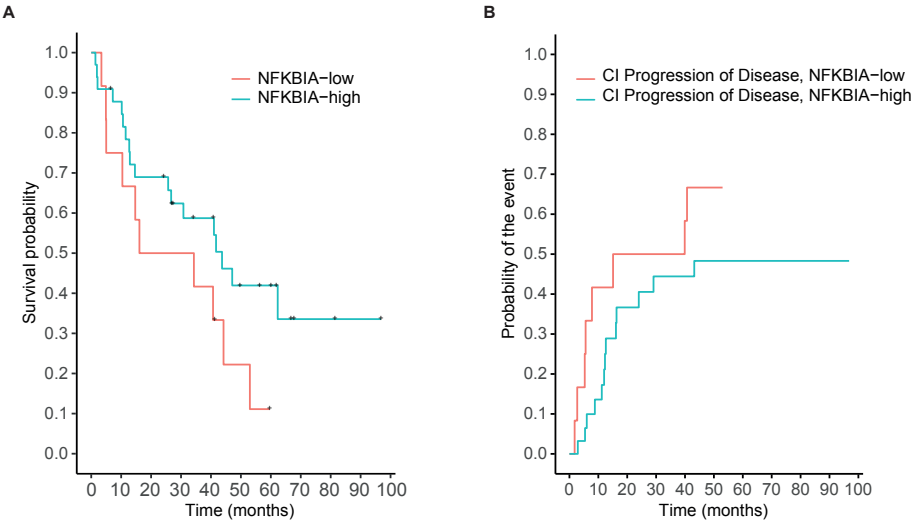
B

Data Sets	Enrichment	Size	NES	FDR
HINATA_NF-kB_TARGETS_FIBROBLAST_UP	NFKBIA High	84	1.87	0.0117
HINATA_NF-kB_TARGETS_KERATINOCYTE_UP	NFKBIA High	91	2.01	0.0038
SCHOEN_NF-kB_SIGNALING	NFKBIA High	34	2.20	0.0009

C



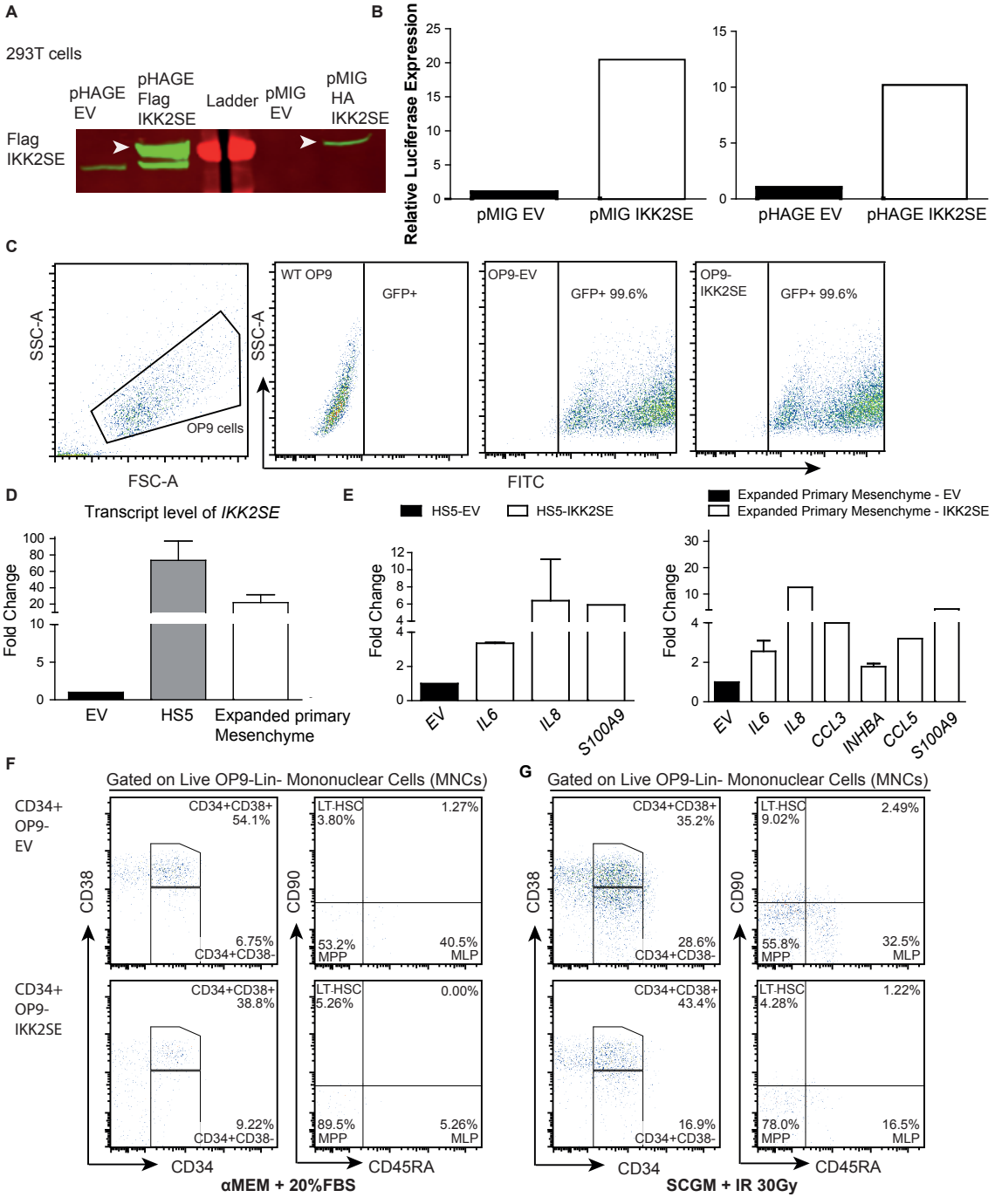
Supplemental Figure 1. Activation of NF-κB signaling in mesenchymal niche cells in a subset of LR-MDS patients. (A) Representative GSEA plots demonstrating enrichment of NF-κB signatures in patients with high (*NFKBIA*$^{+}$) expression (see also figure 1C) in purified mesenchymal cells. The *NFKBIA* cut-off level was arbitrarily defined as mean + 1SD of normal (FPKM 67,93). Other cut-off levels (FPKM 50 or FPKM 107) yielded similar results (data not shown). (B) Listing of enriched NF-κB signatures in *NFKBIA*+ patients. NES: normalized enrichment score; FDR: False Discovery Rate. (C) Expression of *NFKBIA* is significantly correlated to expression of downstream canonical transcriptional targets of NF-κB signaling. Non-parametric correlation estimates (Spearman's rho) and two-sided p-values (α = 0,05) for the relationship between *NFKBIA* expression and *IL6*, *CXCL2*, *CCL3* and *INHBA* are shown. Correlations were obtained using raw FPKM values. P-values were adjusted for multiple testing using the FDR.

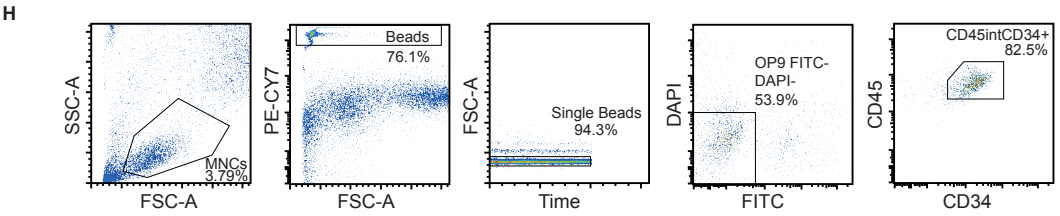


C

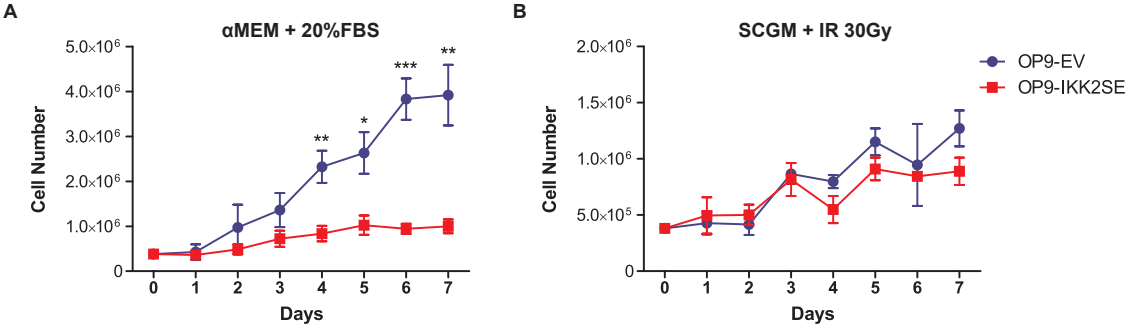
	NFKBIA-low n = 11		NFKBIA-high n = 32		
					P-value
Any mutation	7/11 patients	64%	26/32 patients	81%	0,2477
No mutation	4/11 patients	36%	6/32 patients	19%	
SF3B1	4/11 patients	36%	17/32 patients	53%	
DNMT3A	3/11 patients	27%	5/32 patients	16%	
TET2	2/11 patients	18%	12/32 patients	38%	
SRSF2	1/11 patients	9%	2/32 patients	6%	
ASXL1	1/11 patients	9%	9/32 patients	28%	
RUNX1	1/11 patients	9%	0/32 patients	0%	
IDH2	0/11 patients	0%	2/32 patients	6%	
EZH2	0/11 patients	0%	1/32 patients	3%	
U2AF1	2/11 patients	18%	0/32 patients	0%	
SETBP1	1/11 patients	9%	0/32 patients	0%	

Supplemental Figure 2. Correlation of activated NF-κB signaling in niche cells to clinical outcome and mutational status in LR-MDS. (A) Overall survival probabilities stratified on *NFKBIA* gene expression (low (n = 12) vs. high (n = 33)) were obtained by calculating the Kaplan-Meier estimator. (B) Cumulative incidence curves for progression of disease, stratified on *NFKBIA* low (n = 12) vs. high (n = 31). For two patients, no data was available for the cumulative incidence curves (total n = 43). (C) Mutational status as assessed by gene-panel sequencing. Fisher's Exact Test was used to investigate the difference in proportion of mutations (any vs. no mutation) between *NFKBIA*-low and *NFKBIA*-high (OR = 0.414; 95% CI [0,0713; 2,562]; P = 0.2477). One patient key was unavailable for the *NFKBIA*-low group (total n = 12); one patient of the *NFKBIA*-high group was not sequenced (total n = 33).


A
293T cells
pHAGE EV
pHAGE Flag IKK2SE
Ladder
pMIG EV
pMIG HA IKK2SE
Flag IKK2SE
B
Relative Luciferase Expression
pMIG EV
pMIG IKK2SE
pHAGE EV
pHAGE IKK2SE
C
SSC-A
FSC-A
OP9 cells
WT OP9
GFP+
OP9-EV
GFP+ 99.6%
OP9-IKK2SE
GFP+ 99.6%
FITC
D
Transcript level of IKK2SE
Fold Change
EV
HS5
Expanded primary Mesenchyme
E
HS5-EV
HS5-IKK2SE
Expanded Primary Mesenchyme - EV
Expanded Primary Mesenchyme - IKK2SE
Fold Change
EV
IL6
IL8
S100A9
CCL3
INHBA
CCL5
F
Gated on Live OP9-Lin- Mononuclear Cells (MNCs)
CD34+ OP9- EV
CD34+CD38+ 54.1%
6.75% CD34+CD38-
LT-HSC 3.80%
1.27%
53.2% MPP
40.5% MLP
CD34+ OP9- IKK2SE
CD34+CD38+ 38.8%
9.22% CD34+CD38-
LT-HSC 5.26%
0.00%
89.5% MPP
5.26% MLP
CD38
CD34
CD90
CD45RA
αMEM + 20%FBS
G
Gated on Live OP9-Lin- Mononuclear Cells (MNCs)
CD34+CD38+ 35.2%
28.6% CD34+CD38-
LT-HSC 9.02%
2.49%
55.8% MPP
32.5% MLP
CD34+CD38+ 43.4%
16.9% CD34+CD38-
LT-HSC 4.28%
1.22%
78.0% MPP
16.5% MLP
SCGM + IR 30Gy



Supplemental Figure 3. Activation of NF-κB signaling in OP9, HS5 and expanded primary stromal cells. (A) Western blot analysis showing the overexpression of Flag-IKK2SE transfected 293T cells in comparison to pHage-empty vector (EV) transfected 293T cells. As a reference for the protein size of Flag-IKK2SE, lysates of pMIG EV and pMIG HA-IKK2SE transfected 293T cells were included. (B) Level of NF-**κB** luciferase response in pHage-EV and pHage-Flag IKK2 transfected 293T cells (right panel). NF-**κB** luciferase response in pMIG EV and pMIG HA-IKK2SE transfected 293T cells were included as a positive control. (C) Transduction efficiency of phage EV and pHage-Flag IKK2SE in OP9 cells measured by ZsGreen expression comparing to non-transfected OP9 cells. (D) Expression level of IKK2SE in HS5 and expanded primary mesenchyme transduced with either EV or IKK2SE. (E) Expression level of NF-kB downstream targets and disease-relevant negative regulators of hematopoiesis in HS5 mesenchymal cell lines (left panel) and expanded primary mesenchyme (right panel) transduced with EV or IKK2SE. Fold change relative to EV is presented. (F-G) Representative FACS plots showing the immunophenotypically defined HSPCs subsets after exposure to OP9-EV/IKK2SE in serum containing medium condition (F) and serum free medium condition with stromal layers irradiated at 30 Gy (G). The presented plots were gated on live/OP9-/Lin- mononuclear cells (MNCs). The HSPCs subfractions are defined as: $CD34^{+}CD38^{+}$ progenitor population, $CD34^{+}CD38^{-}$ HSPCs, $CD34^{+}CD38^{-}CD45RA^{+}CD90^{-}$ MLPs (multilymphoid progenitors), $CD34^{+}CD38^{-}CD45RA^{-}CD90^{-}$ MPPs (multipotent progenitors), and $CD34^{+}CD38^{-}CD45RA^{-}CD90^{+}$ LT-HSCs (long-term hematopoietic stem cells). (H) Representative FACS plot demonstrating the cell counting approach by flow-count fluorosphere beads. Information concerning total number of beads and MNCs were obtained during acquiring on LSRII. OP9 cells were gated out from the MNCs by ZsGreen expression and live cells were selected by DAPI staining. CD45 and CD34 antibodies were applied to identify the frequency of $CD34^{+}$ HSPCs.



Supplemental Figure 4. Mesenchymal cell numbers upon activation of NF-κB signaling in different culture conditions. (A) Proliferation curve of OP9-EV/IKK2SE cells in serum containing medium condition (n=4). (B) Proliferation curve of OP9-EV/IKK2SE cells in serum free medium with 30 Gy irradiation condition (n=2). OP9 cells transduced with EV or IKK2SE were re-plated in 24-well plates under conditions described above for 7 days. Where applicable, irradiation was performed at day 0 following re-plating. Each day OP9-EV/IKK2SE cells in corresponding wells were trypsinized, subsequently total cell number was quantified and plotted. Unpaired t-test was performed for statistical analysis *** $P < .001$ ** $P < .01$ * $P < .05$

SUPPLEMENTARY REFERENCES

1. Zambetti NA, Ping Z, Chen S, Kenswil KJ, Mylona MA, Sanders MA*, et al.* Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia. *Cell Stem Cell* 2016 Nov 3; **19**(5)**:** 613-627.
2. Chen S, Zambetti NA, Bindels EM, Kenswill K, Mylona AM, Adisty NM*, et al.* Massive parallel RNA sequencing of highly purified mesenchymal elements in low-risk MDS reveals tissue-context-dependent activation of inflammatory programs. *Leukemia* 2016 Sep; **30**(9)**:** 1938-1942.
3. Doulatov S, Notta F, Eppert K, Nguyen LT, Ohashi PS, Dick JE. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. *Nat Immunol* 2010 Jul; **11**(7)**:** 585-593.
4. Groschel S, Sanders MA, Hoogenboezem R, de Wit E, Bouwman BA, Erpelinck C*, et al.* A single oncogenic enhancer rearrangement causes concomitant EVI1 and GATA2 deregulation in leukemia. *Cell* 2014 Apr 10; **157**(2)**:** 369-381.
5. Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA*, et al.* Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* 2005 Oct 25; **102**(43)**:** 15545-15550.
6. Medyouf H, Mossner M, Jann JC, Nolte F, Raffel S, Herrmann C*, et al.* Myelodysplastic cells in patients reprogram mesenchymal stromal cells to establish a transplantable stem cell niche disease unit. *Cell Stem Cell* 2014 Jun 5; **14**(6)**:** 824-837.
7. van Dieren JM, Simons-Oosterhuis Y, Raatgeep HC, Lindenbergh-Kortleve DJ, Lambers MEH, van der Woude CJ*, et al.* Anti-inflammatory actions of phosphatidylinositol. *Eur J Immunol* 2011 Apr; **41**(4)**:** 1047-1057.
8. Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J*, et al.* IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* 1997 Oct 31; **278**(5339)**:** 860-866.
9. Bosman MC, Schepers H, Jaques J, Brouwers-Vos AZ, Quax WJ, Schuringa JJ*, et al.* The TAK1-NF-kappaB axis as therapeutic target for AML. *Blood* 2014 Nov 13; **124**(20)**:** 3130-3140.

4

UNDER EMBARGO UNTIL PUBLISHED

5

GENERAL DISCUSSION AND CONCLUSION

5.1 SUMMARY

Hematopoiesis is carefully orchestrated by intrinsic programs in the hematopoietic stem and progenitor cells (HSPCs) and extrinsic cues from the bone marrow niche. Disruptions of these delicate programs and cues could result in hematological disorders. Bone marrow failure (BMF) syndromes, including Shwachman-Diamond syndrome (SDS) and myelodysplastic syndrome (MDS), are associated with increased progression to acute myeloid leukemia (AML). The molecular mechanisms underlying the pathogenesis of hematological disorders and leukemogenesis remain incompletely understood. Classically, BMF and leukemogenesis were thought to be solely driven by HSPC intrinsic molecular mechanisms. However, recent experimental findings have suggested that the bone marrow niche is also an important contributor to the initiation and evolution of BMF syndromes. The work described in this dissertation aimed to address two key questions in the field: first, what are the molecular mechanisms underlying the concept of niche driven BMF and leukemogenesis, and second, what is their relevance to human diseases?

The concept of niche-induced oncogenesis has been established in the hematopoietic system. In **chapter 2**, we generated a mouse model with targeted deletion of the Shwachman-Bodian Diamond syndrome (*Sbds*) gene, in *Osterix* expressing mesenchymal progenitor cells (MPCs), to interrogate the underlying mechanism of niche contributions to BMF and leukemogenesis. Mutant mice developed skeletal and myelodysplastic phenotypes recapitulating key features of human SDS. *Sbds*-deficient MPCs induced genotoxic stress in HSPCs, as reflected by mitochondrial hyperpolarization, increased levels of reactive oxygen species (ROS), accumulation of γ-H2AX, and cell-cycle arrest. Activation of the p53 pathway in *Sbds*-deficient MPCs was associated with the skeletal and hematopoietic abnormalities. Transcriptional analysis of mesenchymal cells from SDS, MDS, and Diamond-Blackfan anemia (DBA) patients revealed overexpression of *S100A8* and *S100A9* in comparison to healthy donors. S100a8 and S100a9 proteins were overexpressed in the *Sbds*-deficient MPCs, in line with the increased secretion of S100a8/9 heterodimer in the plasma of the mutant mice. S100a8/9 induced genotoxic stress in HSPCs in both *ex vivo* and *in vivo* assays, in part via TLR4 signaling. However, HSC exhaustion and malignant transformation were not observed in the experimental context of this genotoxic environment. It is possible that the limited survival of the animals prevented HSC transformation. Alternatively, attenuation of DNA repair in genomically altered (i.e. *Sbds* deficient) HSCs may be required. To broaden the relevance of our findings to human disease, RNA sequencing and analysis were performed for the $CD271^+$ MPCs of 45 low-risk MDS (LR-MDS) patients. These patients could be categorized into S100 niche positive and S100 niche negative subpopulations based on the expression levels of *S100A8* and *S100A9* in their $CD271^+$ MPCs. The S100 niche positive patients demonstrated faster leukemic evolution and worse clinical outcome, suggesting a potential value of S100A8/9 as a novel prognostic parameter for LR-MDS.

In addition to the p53-S100A8/9-TLR4 axis, we previously demonstrated the activation of other inflammatory programs in MDS.[1] In **chapter 3**, we assessed the role of NF-κB activation in this context. Transcriptional profiling revealed significantly enriched signatures of the NF-κB pathway and overexpression of *NFKBIA* in the CD271$^+$ MPCs of 45 LR-MDS patients, which was subsequently confirmed at the protein level by immunostaining. To investigate the impact of mesenchymal NF-κB activation on HSPCs, we established stromal cell lines which overexpressed a constitutively active form of IKK2 (IKK2SE), a kinase upstream regulator of NF-κB. Transcriptional upregulation of inflammatory cytokines was observed in IKK2SE expressing stromal cells, recapitulating our findings in MDS patients. Coculture of IKK2SE overexpressing stromal cells with bone marrow HSPCs (CD34$^+$ cells) from healthy donors resulted in attenuated number and function of HSPCs. Our findings provide experimental support for the view that an activated NF-κB inflammatory pathway in mesenchymal cells negatively regulates HSPCs, which might contribute to the molecular pathogenesis of MDS.

In chapter 2 and 3, we focused on the bone marrow niche contribution to BMF syndromes and leukemogenesis. In **chapter 4**, we focused on cytopenia, which is a defining feature of BMF syndromes. Neutropenia is the hallmark of SDS, an inherited BMF syndrome with a high propensity for MDS/AML progression. We tested the hypothesis that cytopenia (neutropenia) may contribute to proliferative stress, exhaustion and possible malignant transformation of HSCs. To study the effect of neutropenia on HSCs and leukemic progression in SDS, we transplanted fetal liver cells with targeted deletion of *Sbds* driven by *Cebpa* expression into lethally irradiated mice. This resulted in severe and persistent neutropenia,[2] even though the immunophenotypic HSCs were genomically intact for *Sbds*. The frequency and absolute number of HSCs were not altered upon primary transplantation, in line with unaltered cell cycle status, levels of ROS and γ-H2AX. Instead, multipotent progenitors (MPPs) were primed to differentiate and compensate for the loss of neutrophils, as demonstrated by increased cell cycle and reduced numbers. RNA sequencing and analysis further supported these observations by revealing enriched transcriptional signatures in oxidative phosphorylation and cell-cycle in MPPs, in comparison to glycolysis and downregulated signatures associated with translation in HSCs under neutropenia. Upon serial transplantations, control HSCs exhibited phenotypes of ageing, whereas HSCs exposed to prolonged neutropenia demonstrated increased competitive repopulation capacity, indicated preservation of function, in particular self-renewal. The exact mechanism of HSC preservation in neutropenia is currently under investigation. However, leukemic transformation was not observed in this mouse model, suggesting that neutropenia *per se* is not sufficient to drive leukemogenesis.

To summarize, the work performed in the scope of this dissertation yielded novel insights into the molecular mechanisms underlying the pathogenesis BMF syndromes. We have discovered that mesenchymal inflammation drives genotoxic stress in HSPCs and predicts disease evolution in human pre-leukemia, that activation of NF-κB driven inflammatory programs in mesenchymal elements attenuates hematopoiesis in LR-MDS and finally, that neutropenia *per se* does not seem to be a contributing factor to HSC attrition. Our findings in the context of recent developments in the field, as well as potential directions for future research will be discussed in the following subchapters.

5.2 NICHE CONTRIBUTIONS TO BMF SYNDROMES: INFLAMMATION TAKES CENTER STAGE

BMF syndromes have been classically considered as hematopoietic cell autonomous disorders, in which HSPC intrinsic mechanisms drive the initiation and progression of these diseases. Only about two decades ago, emerging evidence started to suggest a contribution of the bone marrow niche to the pathogenesis of BMF syndromes. In MDS, the most common form of acquired BMF, bone abnormalities characterized by osteoporosis have been observed in comparison to age-matched controls.[3,4] Moreover, bone marrow derived stromal cells from MDS patients have demonstrated a reduced ability to support HSPCs.[5,6] Last but not least, the occurrence of donor cell-derived hematopoietic neoplasm has suggested a contribution of ancillary cells in the initiation of MDS.[7] Collectively, these data have challenged the view that MDS is solely driven by hematopoietic cell autonomous events, and suggest a role for the bone marrow niche.

Follow-up studies have provided experimental evidence that the bone marrow niche can drive malignant transformation in mouse models.[8,9] In a mouse model of *Dicer1* knockout in *Osterix*-expressing MPCs, mutant mice recapitulated MDS phenotypes, with progression to AML in a subset of animals. Significant downregulation of *Sbds* was observed in the *Dicer1* deficient MPCs.[8] When *Sbds* was deleted in *Osterix*-expressing MPCs, myelodysplasia in the blood and bone marrow, and apoptosis in the HSPCs were observed.[8] However, the molecular mechanisms underlying these phenotypes required further investigation.

The work described in **chapter 2** of this dissertation has provided novel mechanistic insights into niche induced leukemogenesis, and suggested that mesenchymal inflammation plays a central role in this process. We identified S100A8/9 as a key inflammatory molecule in this context. S100A8 and S100A9, also known as alarmins, are damage-associated molecular pattern proteins (DAMPs) involved in modulating inflammation and apoptosis upon encountering danger signals such as infection. The local concentration of S100A8/9 at sites of inflammation can reach significantly higher levels than systemic concentrations detected in the plasma, probably due to the binding to proteoglycans at the local site of secretion.[10-12] S100A9 has been shown to promote myelopoiesis via the induction of ROS.[13] In line with our findings, Schneider *et al.* have demonstrated that *Rps14* haploinsufficiency leads to p53 activation and S100a8/9 overexpression in macrophages and erythroid cells, which induced apoptosis in erythroid progenitors and defects in erythroid differentiation, in a mouse model of del(5q) MDS.[14] Furthermore, another study has demonstrated that somatic mutations in HSPCs from LR-MDS patients manifested NLRP3 inflammasome activation with overexpression of inflammatory cytokines including S100A9 and IL-1β, leading to ROS and pyroptotic death in HSPCs.[15]

Altogether, we have demonstrated that mesenchymal inflammation contributes to the pathogenesis of human BMF syndromes and identified S100A8/9 as a key inflammatory molecule overexpressed in the bone marrow niche to drive disease phenotypes. Converging literature regarding S100A8/9 in different mouse models and patient samples has corroborated these findings, suggesting S100A8/9 is an important molecule for the pathogenesis of MDS. In the light of this novel finding, several new questions emerged, concerning the driver of mesenchymal S100A8/9 overexpression, and its relevance to BMF and leukemogenesis, which will be discussed in the following paragraphs.

5.2.1 Mesenchymal inflammation in BMF: chicken or egg?

The tumor suppressor p53 is a known upstream driver of S100A8 and S100A9 expression.[16] In **chapter 2**, we have identified a novel mechanism, in which targeted deletion of *Sbds* in *Osterix*-expressing MPCs resulted in p53 upregulation and drove the mesenchymal overexpression of S100a8/9. Moreover, targeted deletion of *Trp53* in *Sbds* deficient *Osterix*-expressing MPCs rescued the bone phenotype as well as the genotoxic stress in HSPCs, demonstrating that p53 activation in mesenchymal cells is upstream of *S100a8/9* overexpression in this model. In line with our finding, Schneider *et al*. have demonstrated that the overexpression of S100a8 in hematopoietic cells is also regulated by p53.[14]

Whether the mesenchymal overexpression of S100A8/9 is caused by primary alterations in mesenchymal cells or induced by primary events in aberrant hematopoietic cells is debatable. The answer to this question may be context dependent: in congenital BMF syndromes such as SDS, the germline mutations in MPCs can drive disease associated phenotypes. However, in acquired BMF syndromes such as MDS, it is more likely that acquired genetic events in hematopoietic cells are the initiating event.

Mutations in MPCs might propagate to their downstream progeny in the bone marrow cavity, creating a mutagenic microenvironment for HSPCs and inducing pathogenesis. This scenario may be relevant to congenital BMFs in which the mutation is already present during embryonic and perinatal development. In contrast, it is probably not common in acquired BMF, because a *de novo* mutation in the mesenchymal niche is unlikely to propagate and affect a large area in the bone marrow cavity of patients.[17] Although distinct cytogenetic abnormalities have been identified in *ex vivo* expanded mesenchymal cells in MDS, it is still unclear whether these abnormalities are present *in situ* or caused by serial *ex vivo* expansion.[18,19] Moreover, these proposed cytogenetic abnormalities in MDS mesenchymal cells have not been clearly linked to pathogenetic mechanisms of niche-instructed disease phenotypes.

Alterations in hematopoietic cells are generally considered as the primary event in acquired BMF syndromes, which may initiate inflammatory signaling in the mesenchymal niche and

subsequently support leukemic progression.[17] A previous study of our group has compared freshly isolated and *ex vivo* expanded CD271$^+$ MPCs from MDS.[1] Transcriptome analysis revealed that many inflammatory programs are tissue context dependent, suggesting the interaction between mesenchymal cells and hematopoietic cells is important for the maintenance of inflammatory signatures in MDS.[1] In line with this, coculture of healthy mesenchymal stromal cells with MDS bone marrow resulted in alterations of the transcriptome towards MDS-like transcriptome, indicating that MDS HSPCs are capable of instructing transcriptional alterations in surrounding mesenchymal cells, which may further reprogram the niche to support MDS HSPCs and drive disease phenotypes.[20] Importantly, a recent study employing a robust mouse model of del(5q) MDS demonstrated that genetic alteration in HSPCs leads to S100a8 overexpression in macrophages, which induces S100a8 expression in MSCs to further impair hematopoiesis.[21] In line with this observation, S100A8 overexpression was also confirmed in the CD271$^+$ MPCs of del(5q) MDS patients in comparison to healthy donors.[21]

Collectively, our findings suggest that the mesenchymal upregulation of S100a8/9 in SDS is driven by p53 activation in MPCs, which could be implicated in the molecular pathogenesis of other congenital BMF syndromes. There is currently insufficient experimental evidence that primary alterations in mesenchymal cells are the driver of disease pathogenesis in acquired human BMF syndromes.

5.2.2 Can S100A8/9 overexpression contribute to different aspects of the MDS phenotype?

MDS is characterized by ineffective hematopoiesis, cytopenia (in particular anemia) and increased risk for AML progression. It is tempting to speculate that distinct aspects of MDS phenotypes are driven by similar molecular mechanisms. We have identified the overexpression of S100A8/9 in mesenchymal cells is involved in MDS pathogenesis. In other studies, the overexpression of S100A8/9 in different cell types have been observed.[13-15]

The bone marrow microenvironment is composed of highly complex structures, specific niche components support distinct HSPCs: erythroblastic islands have been identified as the niche for erythroid progenitors, whereas MSCs have been identified as the niche for HSCs.[22,23] Schneider *et al*. have demonstrated that S100a8/9 overexpression in erythroblastic islands drives anemia,[14] which is a common phenotype in LR-MDS patients. Upregulation of S100a9 protein in hematopoietic cells has been reported to induce the accumulation of myeloid-derived suppressor cells (MDSCs), which play pathogenic roles in MDS.[13,24] In addition, Brasiorka *et al*. have demonstrated that upregulation of S100A9 in HSPCs resulted in elevated ROS and genotoxic stress.[15] Finally, we have identified S100A8/9 overexpression in MPCs drives myelodysplasia and genotoxic stress in HSCs, which is associated with increased propensity to AML progression in LR-MDS patients.

Direct versus indirect effects of mesenchymal cells on HSPCs

Our findings have demonstrated the direct effects of mesenchymal S100A8/9 overexpression on HSPCs. In addition, indirect effects may be at play. Mesenchymal overexpression of S100A8/9 may recruit other innate immune cells to affect the function of HSCs, which might exaggerate the inflammatory microenvironment and foster the disease-relevant phenotypes. In line with this idea, we have observed the accumulation of S100a8/9 expressing myeloid cells at the bone/marrow interface of *Osterix*-cre *Sbds*$^{F/F}$ mice (data not shown). Additional experimental evidence has been provided in the context of Noonan syndrome, which is a congenital disorder associated with skeletal abnormality and leukemia predisposition.[25] Genetically engineered mice carrying an activating mutation of *Ptpn11* in mesenchymal cells, produce excessive CCL3 to recruit monocytes, which create an inflammatory microenvironment via the secretion of IL-1β, leading to hyperactivation of HSCs and development of myeloproliferative neoplasm (MPN).[25]

Taken together, our findings, in concert with recent converging literature, suggest that S100A8/9 overexpression in distinct niches and cell types may drive different aspects of MDS pathogenesis. The overexpression of S100A8/9 in erythroblastic islands may drive anemia, whereas its overexpression in MPCs may drive myelodysplasia and genotoxic stress in HSCs. Multiple sources of S100A8/9 might be involved, and synergistically contribute to the pathogenesis of MDS. Genetic mouse models harboring cell type specific knockout of *S100a8/9* may be a valuable tool to verify this notion.

5.2.3 What is the link between mesenchymal S100A8/9 overexpression and leukemogenesis? Induction versus selection of preleukemic clones

SDS and MDS are BMF syndromes with strong propensity for leukemic evolution. We have demonstrated that the targeted deletion of *Sbds* in *Osterix*-expressing MPCs induced overexpression of S100a8/9 and led to genotoxic stress in HSCs. Notably, leukemogenesis was not observed in this mouse model: the limited follow-up period of 4 weeks may have precluded the latency of genetic events required for malignant transformation. Alternatively, it is conceivable that the mutagenic niche leads to leukemic progression only in the context of DNA-repair deficient HSPCs. Nevertheless, the pathogenetic link between mesenchymal niche inflammation, genotoxic stress in HSPC, and leukemic evolution in SDS and MDS merits further discussion on the putative leukemogenic effects of S100A8/9 mediated inflammatory signaling.

One potential mechanism could be that the genotoxic stress induced by S100A8/9 overexpressing niche cells leads to the accumulation of mutations in HSCs and, eventually, leukemic progression. The overexpression of matrix metallopeptidase-3 (MMP-3) has been shown to induce ROS, which leads to DNA damage and genomic instability in the

microenvironment of breast cancer.[26,27] The clinical evidence, however, is not consistent with a 'hypermutator' phenotype of HSPCs in SDS, as no increase of mutation burden was observed in the HSPCs of SDS patients.[28] Collectively, this evidence may argue against the induction of genetic events in HSPCs by a mutagenic niche as the key driver of leukemogenesis, at least in the context of SDS.

Another potential mechanism could be that the S100A8/9 overexpressing mutagenic niche provides a hostile environment, in which HSCs carrying specific mutations survive and gain a competitive advantage over normal HSCs. Loss-of-function mutations in genes encoding epigenetic regulators, including *DNMT3A*, *ASXL1*, and *TET2* have been characterized as the most frequently occurring mutations in the HSCs of MDS patients.[29] These mutation-bearing HSCs are commonly defined as pre-leukemic HSCs.[30] The progression of MDS to AML has been proposed to begin with the selection and expansion of pre-leukemic HSC clones.[31] The mutagenic microenvironment might facilitate the sequential acquisition of additional somatic mutations in pre-leukemic clones over time, eventually leading to full-blown AML. The molecular mechanism underlying the survival, engraftment, and expansion of pre-leukemic HSCs remains largely elusive; inflammatory signaling might play an essential role in these processes. Emerging studies have started to illustrate how pre-leukemic HSCs respond to inflammation.[32,33] For example, a recent publication has demonstrated the resistance of *Tet2* knockout HSPCs to acute inflammatory insult induced by LPS, and identified a role for IL-6 in promoting the survival and functionality of *Tet2* deficient HSPCs.[34] IL-6 is one of the most prominent inflammatory cytokines circulating in the blood, which has a broad effect on hematopoiesis and has been implicated in leukemic transformation.[35] It is thus conceivable to envision a model in which mutated hematopoietic cells create an inflammatory environment, favoring their own propagation at the cost of diminishing wildtype competitors.

To provide more mechanistic insights into the link between mesenchymal S100A8/9 overexpression and leukemogenesis, several follow-up experiments could be proposed. Transplanting hematopoietic cells carrying a deletion in genes frequently mutated in MDS, such as *Dnmt3a*, *Asxl1*, *Tet2*, and *Trp53*, into *S100a9* transgenic mice, might be helpful to reveal the underlying mechanism in which pre-leukemic HSCs react to genotoxic stress induced by mesenchymal S100a8/9 overexpression. Moreover, it would be helpful to generate a hypomorphic mouse model of SDS, by intercrossing the *Osterix*$^{cre/+}$ *Sbds*$^{F/+}$ mouse to *Sbds*$^{R126T/+}$ mouse, in which a human disease-relevant mutation of *Sbds* is introduced.[36] The resulting *Osterix*$^{cre/+}$ *Sbds*$^{F/R126T}$ mice might have a less severe phenotype associated with prolonged life-span, enabling an extended follow-up period, allowing for the transplantation of pre-leukemic HSCs and the study of leukemic progression.

Altogether, a hypothetical model of mesenchymal niche inflammation driven BMF and leukemogenesis can be proposed (Figure 1). The inflammatory signaling may initiate in the niche or in the hematopoietic cells, leading to a microenvironment with excessive amounts of inflammatory molecules. These inflammatory molecules may act on HSCs either directly via receptors, or indirectly via recruiting innate immune cells, forming a vicious circle. The inflammatory microenvironment may cause genotoxic stress in HSPCs resulting in BMF. At the same time, facilitating the selection of pre-leukemic HSC clones by providing protection against mutagenic stress, at the expense of poising them for additional mutations, eventually leading to full-blown AML.

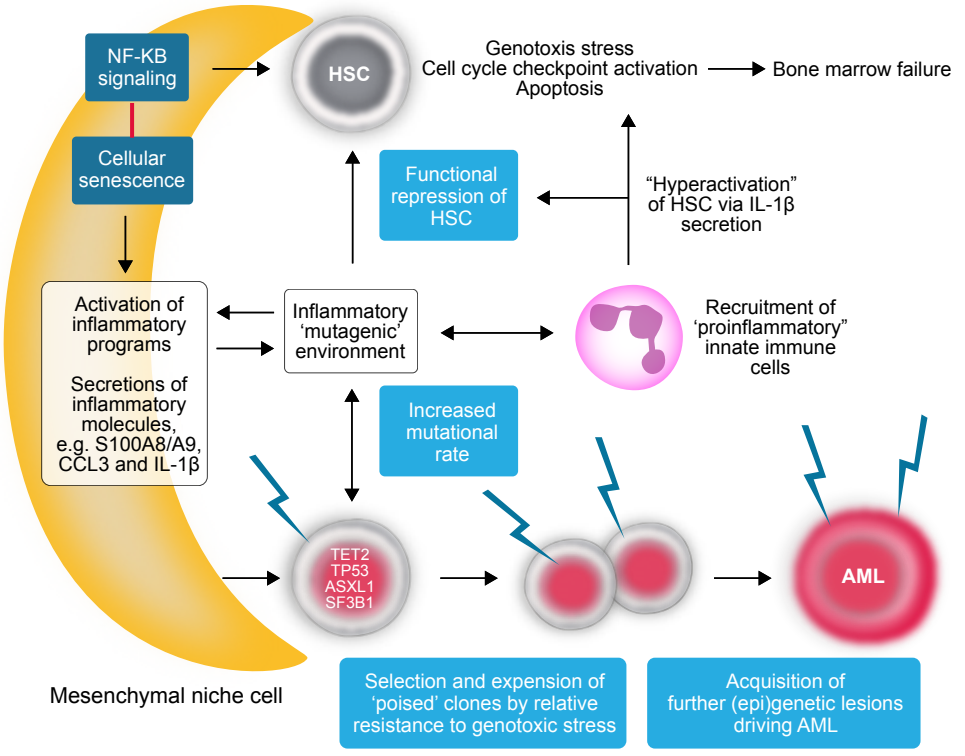


Figure 1. A hypothetical model of mesenchymal niche inflammation driven leukemic progression in BMF syndromes. Adapted from Pronk and Raaijmakers.[17] Disruption of molecular programs in mesenchymal niche cells may lead to the secretion of inflammatory molecules to create a mutagenic environment, which could further recruit proinflammatory innate immune cells. This mutagenic niche may contribute to the pathogenesis of BMF syndromes by inducing genotoxic stress in HSCs, or facilitating the selection and expansion of pre-leukemic HSCs, eventually resulting in leukemia. The involved cellular elements, signaling pathways, inflammatory molecules, and genetic mutations are annotated.

In conclusion, our findings **in chapter 2** in a monogenic model of congenital BMF provide experimental evidence that mesenchymal inflammation plays a central role in the pathogenesis of BMF syndromes. We identified S100A8/9 as a key inflammatory molecule which induces genotoxic stress in neighboring HSPCs. In acquired MDS, the overexpression of S100A8/9 is likely to start in hematopoietic cells and subsequently spread to mesenchymal cells. Different aspects of MDS phenotype may be driven by S100A8/9 overexpression in distinct HSPC niches. The inflammatory microenvironment may facilitate the selection of pre-leukemic HSCs for leukemic progression. Future experiments would be required to address which hematopoietic cell subsets are cross-talking to the mutagenic niche, and whether mutation specific signaling is involved.

5.2.4 Mesenchymal niche inflammation and its upstream drivers in BMF syndromes: beyond S100A8/A9

Inflammation has been extensively associated with cancer and speculated to be an enabling factor in malignancies.[37] S100a8/9 was not the only inflammatory molecule upregulated in the *Sbds* deficient *Osterix*-expressing MPCs in our mouse model; genes encoding for inflammatory cytokines, such as Ccl3, IL-1β, and IL-6, were also overexpressed (data not shown). In line with this observation, transcriptional profiling of human CD271$^+$ MPCs has revealed the overexpression of *CCL3* and *IL-1β* in MDS patients compared to healthy controls.[1] Interestingly, many of these molecules are downstream targets of NF-κB, which is a master upstream regulator of inflammation.[38,39] Activation of NF-κB signaling in HSPCs has been demonstrated to cause BMF.[40] However, much less has been reported regarding the role of mesenchymal NF-κB signaling in the regulation of hematopoiesis.

NF-κB as a master upstream driver of mesenchymal inflammation

NF-κB is probably one of the most well-documented transcription factors involved in immune regulation, inflammation, and cancer. Consisting of five monomers, the NF-κB family of transcription factors can yield fifteen dimer combinations to activate or repress hundreds of target genes involved in a wide spectrum of cellular processes.[39] The activation of canonical NF-κB pathway is mediated by the phosphorylation and subsequent degradation of a family of proteins called inhibitor of κB (IκB), which leads to the nuclear localization of NF-κB to regulate gene expression.[38] IκB is specifically phosphorylated by a kinase complex called IκB kinase (IKK).[41] NF-κB is known to induce the expression of IκB as a negative feedback loop.[42]

In **chapter 3**, we have reported the upregulation of *NFKBIA* (which encoded IκBα, a member of the IκB family) in the CD271$^+$ MPCs in a subset of LR-MDS patients; the expression of *NFKBIA* significantly correlated to the expression of canonical NF-κB downstream targets, including *CCL3*, *IL6*, *CXCL2*, and *INHBA*. To experimentally mimic the mesenchymal activation of NF-κB, we experimentally overexpressed a constitutively active form of IKK2 (also known

as IKKβ, a kinase subunit of IKK), *IKK2SE*,[43] in OP9 stromal cells. Upon the overexpression of *IKK2SE*, we observed a significant upregulation of *NFKBIA* and many NF-κB downstream targets, including *Ccl3* and *IL6* in OP9 cells, consistent with our earlier observation in the CD271$^+$ MPCs of MDS patients.[1] Many of these inflammatory molecules have been identified as negative regulators of hematopoiesis, suggesting that their mesenchymal overexpression could also be implicated in the pathogenesis of MDS.[25,34] Altogether, our findings suggest NF-κB as a master upstream regulator of mesenchymal inflammatory signaling implicated in the pathogenesis of MDS.

Primary alterations in hematopoietic cells could be a cause of NF-κB activation in mesenchymal cells. Coculture of MPN or MDS bone marrow with healthy mesenchymal stromal cells (MSCs) has been demonstrated to induce transcriptional and functional alterations in MSCs to favor the expansion of diseased hematopoietic cells. Inflammatory programs have been implicated in these MSC alterations, but their relevance to NF-κB signaling requires further investigation.[20,44] Moreover, enriched transcriptional signatures of inflammation have been reported in FACS-purified MPCs compared to *ex vivo* expanded MPCs from LR-MDS patients, suggesting that hematopoietic cells might be implicated in causing these alterations.[1] Besides, we have cocultured LR-MDS bone marrow with OP9 stromal cells and observed an upregulation of *NFKBIA* compared to OP9 cells alone (data not shown).

A potential role for cellular senescence warrants further discussion in this context. Cellular senescence could be both a cause and a consequence of mesenchymal NF-κB activation. We have demonstrated that activation of NF-κB signaling led to decreased proliferation, a flat morphology and enlarged nuclei in OP9 stromal cells, reminiscent of a senescence phenotype. In addition, the expression of *NFKBIA* correlated with the expression of *p21*, a mediator of cellular senescence,[45] in the CD271$^+$ MPCs of LR-MDS patients (data not shown). Senescence could induce the secretion of inflammatory molecules which are tumor-promoting, collectively known as the senescence-associated secretory phenotype (SASP).[46,47] NF-κB has been demonstrated to be a master regulator promoting SASP.[48,49] The upregulation of *IL-6*, a key component of the SASP, among many other inflammatory molecules, was observed following the activation of NF-κB in OP9 stromal cells. Moreover, senescence has been associated with decreased MSC support for hematopoiesis in MDS patients.[50,51] Consistently, we have observed attenuation in the number and function of HSPCs upon coculture with OP9 stromal cells with constitutive NF-κB activation. However, to formally establish whether mesenchymal senescence is a cause or consequence of NF-κB activation in our model, further examination of senescence markers, including p16^{Ink4A} and senescence-associated beta-galactosidase are required in the IKK2SE overexpressing OP9 stromal cells.

In conclusion, our findings in **chapter 3** demonstrate that mesenchymal NF-κB activation drives inflammatory signaling and attenuates HSPCs in LR-MDS patients. The upregulation of inflammatory molecules driven by NF-κB activation is likely to create a hostile microenvironment which attenuates HSPC number and function. The activation of NF-κB is likely to be induced by primary alterations in hematopoietic cells. Cellular senescence could be a cause or consequence of NF-κB activation, although further experimental evidence is required to support this hypothesis.

5.3 CYTOPENIA AS A DRIVER OF HSC ATTENUATION IN BMF SYNDROMES

The pathogenesis and leukemic progression of BMF syndromes are driven by molecular events both in the hematopoietic cells and the bone marrow niche. In **chapter 4**, we looked at cytopenia, defined by reduced numbers in one or multiple lineages of mature cells in the blood, which is a hallmark of BMF syndromes associated with high propensity for leukemic progression. Here, we asked the question whether cytopenia *per se* might contribute to malignant transformation of HSCs in BMF syndromes such as SDS, severe congenital neutropenia (SCN), and MDS.

Neutropenia as a driver of HSC exhaustion and leukemic transformation in SDS?

Neutropenia is a hallmark of SDS, a disease in which patients have high propensity to develop MDS/AML at a young age.[52,53] In **chapter 4**, we investigated how HSCs respond to prolonged neutropenia and its relevance to leukemic progression. Our original hypothesis was that upon prolonged exposure to neutropenia, HSCs respond by entering the cell cycle and thereby accelerating the production of downstream progenitors to compensate for the loss of neutrophils. During this process, HSCs could acquire replicative stress, which drives the accumulation of ROS and DNA damage. These events may further lead to HSC exhaustion, restriction of the HSC pool size, and increased replicative stress in a self-enforcing circuitry, which ultimately result in the collapse of the hematopoietic system with leukemic transformation, perhaps as an 'escape' mechanism.

We tested our hypothesis in a robust SDS mouse model of neutropenia generated earlier in our lab, by serially transplanting hematopoietic cells carrying a targeted deletion of *Sbds* driven by *Cebpa* expression.[2] In this model, neutrophil differentiation is impaired at the myelocyte stage, resulting in pronounced neutropenia, while the vast majority of HSCs remain genetically intact (*Sbds* proficient). We observed profound neutropenia 4 weeks after each transplantation, which was sustained in 3 serial transplantations spanning a total follow-up period of 18 months. Importantly, the immunophenotypic HSCs in our mouse model had intact *Sbds* as demonstrated by transcriptome analysis.

However, contrary to our original hypothesis, the immunophenotypic HSC pool did not expand upon exposure to neutropenia and did not exhibit replicative stress. Cell cycle status was unaltered and transcriptional Myc targets were downregulated. HSCs exposed to neutropenia were metabolically inactive, as suggested by Gene Set Enrichment Analysis (GSEA) signatures of glycolysis and reduced mitochondria-derived ROS levels. In line with this, no signatures of DNA damage and repair (DDR) pathways, and no change in γ-H2AX levels were found. Furthermore, no leukemic transformation was observed during the total follow-up period of serial transplantation. Altogether, we found no experimental evidence

supporting the view that neutropenia *per se* contributes to HSC exhaustion and leukemic transformation.

Rather, our data suggested that the integrity and function of HSCs were preserved in neutropenia. In line with this, upon tertiary transplantation, HSCs exposed to prolonged neutropenia exhibited increased repopulation capacity compared to HSCs of control recipients.

Sparing of multipotent quiescent HSCs by preferential engagement of myeloid primed transit-amplifying cells

One potential explanation for this unanticipated finding could be that neutropenia is coped with differentiation of specific myeloid biased HSCs and expansion of transit-amplifying cells, sparing the *bona fide* multipotent quiescent HSC population. In line with the unchanged number and metabolically inactive phenotypes of immunophenotypic HSCs observed in our neutropenia model, a recent publication has demonstrated that HSCs can differentiate into restricted myeloid progenitors without undergoing cell division.[54] In addition, transcriptional profiling revealed that HSCs exposed to neutropenia were primed for myeloid differentiation, as supported by enriched myeloid differentiation signatures and upregulation of myeloid commitment genes. In contrast, the number of MPPs was significantly decreased upon exposure to neutropenia. These MPPs demonstrated increased proliferation and gave rise to the dramatically expanded myeloid-biased progenitors including HPC1 and HPC2, to compensate for the loss of mature neutrophils. In line with this, enriched GSEA signatures of oxidative phosphorylation, Myc targets, mitotic cell cycle, and myeloid development signatures were observed in MPPs, suggesting that MPPs are the main drivers of the response to neutropenia. While this coping of neutropenia by stem cell differentiation, but not proliferation, could explain maintenance of true HSCs in neutropenia, it does not seem to explain why HSCs in neutropenia actually perform better (increased serial repopulating ability). To explain this, several hypotheses can be postulated.

Active preservation of HSC quiescence in neutropenia?

It is conceivable that the progeny of HSCs signals back to their predecessors in order to protect and augment the long-term repopulating HSC pool and secure homeostasis.[55,56] HSCs exposed to neutropenia might be actively protected by soluble factors secreted either by hematopoietic cells or the bone marrow niche. For example, it has been demonstrated that myeloid-biased HSCs are actively maintained by histamine secreted by their downstream progeny, the bone marrow myeloid cells.[56] Depletion of histamine-producing cells resulted in rapid loss of quiescent state and exhaustion in these myeloid-biased HSCs.[56] The expression

of histamine in downstream myeloid progenitor cells and its secretion into the bone marrow supernatant still need to be examined, to conclude whether a similar mechanism was involved in our experimental model. Notably, expression levels of *Hdc*, the enzyme directly associated with histamine production, were substantially elevated in HSCs and MPPs in neutropenia, suggesting the possibility of enhanced autocrine/paracrine signaling.

Moreover, several hematopoietic cytokines, including M-CSF may be implicated in our model. M-CSF has been demonstrated to instruct myeloid cell fate in mouse HSCs uncoupled to proliferation and survival,[57] suggesting that M-CSF could be involved in skewing the fate of HSCs towards the myeloid lineage without causing proliferative stress. In our model, we did not observe any difference in the levels of M-CSF in plasma and bone marrow supernatant; however, the transcriptional expression levels of M-CSF receptor (M-CSFR) were upregulated in distinct HSPC populations in neutropenia. In future experiments, it would be important to confirm the expression of M-CSF receptor at the protein level, and test whether M-CSF signaling, in addition to myeloid priming, can actively preserve HSCs.

In addition, bone marrow niche populations may indirectly contribute to enhanced HSC function in neutropenia. For example, short-term depletion of peripheral neutrophils has been shown to cause the expansion of mesenchymal niche (CAR cells).[58] In line with that, we also observed an increased frequency of MSCs in our neutropenia mouse model. Moreover, the transcriptional landscape of MSCs was substantially modified upon neutropenia exposure. We have observed significant upregulation of candidate HSC regulating ligands, including *Sparcl-1*, in MSCs and osteoblasts exposed to neutropenia (data not shown). Further validation is required to confirm the overexpression of these factors at the protein level in the mesenchymal cells exposed to neutropenia. Subsequently, the effect of these factors on HSPCs should be robustly addressed in cell culture assays and in transgenic mouse models.

Neutrophils as negative regulators of HSCs and their niche?

In addition to the autocrine/paracrine feedback of positive HSC regulators, withdrawal of negative HSC regulators could also contribute to the mechanism of HSC preservation. Neutrophils are key regulators of inflammation and emerging findings suggest that neutrophils may have a predominantly negative effect on HSC function in the bone marrow. Neutrophils are known to express and release interferons (IFNs) under certain conditions.[59,60] IFN signaling is a main driver of HSC activation and exhaustion in mammals.[61-63] Intriguingly, we found enrichment of multiple IFN downstream signaling signatures in the control HSCs. Significant downregulation of IFN targets, including *Iigp1* was observed in HSCs exposed to neutropenia, which is a key regulator of cellular response to IFNs.[64] It may be speculated that neutrophil-derived IFN release has a long-term negative effect on HSCs. In future

experiments, it would be interesting to examine the role of IFNs secreted by neutrophils in attenuating HSCs. *Ex vivo* exposure of HSCs to neutrophils and subsequent *in vitro* or *in vivo* assay of HSC function may provide experimental evidence to support the notion that neutrophils are direct negative regulators of HSCs.

It is noteworthy that the ageing phenotypes of HSCs, in particular the decline in repopulation capacity and expansion of immunophenotypic HSCs,[65] seemed to diminish in neutropenic mice. Increased myeloid output and enhanced inflammatory signals in ageing bone marrow have been reported, including S100A8/9, of which neutrophils are a major source.[66,67] It is therefore tempting to speculate that neutrophils may contribute to HSC ageing, perhaps through enhanced IFN or S100A8/A9 signaling; this hypothesis may be tested in future experiments.

Nevertheless, it is important to underline that our observations were made in a specific mouse model of *Sbds* deletion in *Cebpa* expressing cells. Although the resulting neutropenia phenotypically recapitulates the neutropenia observed in leukemia predisposition disorders, including SDS and SCN, as characterized by impaired myeloid differentiation at the myelocyte stage, further investigations will have to establish whether these findings can be translated to other conditions of neutropenia/cytopenia. Additional mouse models will be informative, but it is challenging to generate genetic models of neutropenia while sparing the HSC population. Future studies in which neutrophil depletion is caused by the knockout of genes other than *Sbds*, such as *SerpinB1*,[68] in *Cebpa*-cre mice are considered. Moreover, *Lysozyme 2*-cre diphtheria toxin A (DTA) mice may be helpful, but carry the disadvantages of artificial DTA-mediated neutrophil depletion and short-term neutropenia.[69] Finally, immunophenotyping and transcriptional profiling of HSPCs in patients suffering from other hematological disorders associated with neutropenia/cytopenia and leukemic evolution, such as MDS and idiopathic aplastic anemia, could also provide additional insights.

In conclusion, our findings in **chapter 4** suggest that neutropenia *per se* does not lead to cellular stress, HSC exhaustion and leukemic progression in the context of SDS. Rather, our data demonstrated the preservation of HSCs under prolonged exposure to neutropenia. The mechanism through which neutropenia enhances HSC function warrants further elucidation. One possibility is the enrichment of downstream progenitors as positive HSC regulators; another possibility is the withdrawal of neutrophils as negative HSC regulators. Future experimental evidence supporting this new hypothesis may provide further understanding of hematopoiesis.

5.4 CONCLUSIONS AND PERSPECTIVES

Hematopoiesis is an elegantly orchestrated process involving molecular programs in both the HSPCs and the bone marrow niche. Disruptions of these molecular programs could result in hematological diseases, including BMF syndromes and leukemia. The work performed in this dissertation aimed to answer two fundamental questions: what are the molecular mechanisms underlying the concept of niche driven BMF and leukemogenesis, and what is their relevance to human diseases.

In **chapter 2**, we have discovered that mesenchymal upregulation of S100A8/9 induces genotoxic stress in HSPCs and predicts leukemic evolution and disease outcome in human MDS. Our findings provided a novel predictor for LR-MDS based on the mesenchymal expression of S100A8/9, which is independent of the current prognostic factors. Small molecule inhibitors blocking the binding of S100A8/9 heterodimer to its receptor are under clinical development;[70-72] in the future, they may provide a new therapeutic option for the MDS patients with mesenchymal S100A8/9 signatures.

In **chapter 3**, we have demonstrated that activation of NF-κB signaling in mesenchymal cells is common in LR-MDS, leads to transcriptional overexpression of inflammatory factors and attenuates HSPCs. Our findings support the notion that NF-κB as a master driver of mesenchymal inflammation in LR-MDS may be therapeutically targeted in addition to hematopoietic cell specific therapeutics. Nevertheless, the wide spectrum of NF-κB signaling in cellular processes makes its pharmaceutical targeting complicated.[39]

In **chapter 4**, we have observed that neutropenia *per se* does not drive cellular stress in HSCs and leukemic progression, rather, preserves HSCs from ageing and function decline. The molecular mechanism of HSC preservation under neutropenia is currently under investigation. Our findings suggest that the disruption of a single lineage in a multicellular organism does not lead to stem cell exhaustion and does not jeopardize the entire system.

Our discoveries collected in this dissertation have provided novel mechanistic insights into the molecular pathogenesis of BMF syndromes. Further unraveling the interaction between the hematopoietic cells and the bone marrow microenvironment is anticipated to open new avenues for the prevention and treatment of hematological disorders.

REFERENCES

1. Chen, S.*, et al.* Massive parallel RNA sequencing of highly purified mesenchymal elements in low-risk MDS reveals tissue-context-dependent activation of inflammatory programs. *Leukemia* **30**, 1938-1942 (2016).
2. Zambetti, N.A.*, et al.* Deficiency of the ribosome biogenesis gene Sbds in hematopoietic stem and progenitor cells causes neutropenia in mice by attenuating lineage progression in myelocytes. *Haematologica* **100**, 1285-1293 (2015).
3. Mellibovsky, L.*, et al.* Bone remodeling alterations in myelodysplastic syndrome. *Bone* **19**, 401-405 (1996).
4. Weidner, H.*, et al.* Myelodysplastic syndromes and bone loss in mice and men. *Leukemia* **31**, 1003-1007 (2017).
5. Raaijmakers, M.H. Myelodysplastic syndromes: revisiting the role of the bone marrow microenvironment in disease pathogenesis. *International journal of hematology* **95**, 17-25 (2012).
6. Li, A.J. & Calvi, L.M. The microenvironment in myelodysplastic syndromes: Niche-mediated disease initiation and progression. *Experimental hematology* **55**, 3-18 (2017).
7. Flynn, C.M. & Kaufman, D.S. Donor cell leukemia: insight into cancer stem cells and the stem cell niche. *Blood* **109**, 2688-2692 (2007).
8. Raaijmakers, M.H.*, et al.* Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* **464**, 852-857 (2010).
9. Kode, A.*, et al.* Leukaemogenesis induced by an activating beta-catenin mutation in osteoblasts. *Nature* **506**, 240-244 (2014).
10. Vogl, T.*, et al.* Alarmin S100A8/S100A9 as a biomarker for molecular imaging of local inflammatory activity. *Nature communications* **5**, 4593 (2014).
11. Frosch, M.*, et al.* Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. *Arthritis and rheumatism* **43**, 628-637 (2000).
12. Achouiti, A.*, et al.* Myeloid-related protein-14 contributes to protective immunity in gram-negative pneumonia derived sepsis. *PLoS pathogens* **8**, e1002987 (2012).
13. Cheng, P.*, et al.* Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. *The Journal of experimental medicine* **205**, 2235-2249 (2008).
14. Schneider, R.K.*, et al.* Rps14 haploinsufficiency causes a block in erythroid differentiation mediated by S100A8 and S100A9. *Nature medicine* **22**, 288-297 (2016).
15. Basiorka, A.A.*, et al.* The NLRP3 inflammasome functions as a driver of the myelodysplastic syndrome phenotype. *Blood* **128**, 2960-2975 (2016).
16. Li, C.*, et al.* A novel p53 target gene, S100A9, induces p53-dependent cellular apoptosis and mediates the p53 apoptosis pathway. *The Biochemical journal* **422**, 363-372 (2009).
17. Pronk, E. & Raaijmakers, M. The mesenchymal niche in MDS. *Blood* **133**, 1031-1038 (2019).
18. Ramakrishnan, A., Awaya, N., Bryant, E. & Torok-Storb, B. The stromal component of the marrow microenvironment is not derived from the malignant clone in MDS. *Blood* **108**, 772-773 (2006).
19. Lopez-Villar, O.*, et al.* Both expanded and uncultured mesenchymal stem cells from MDS patients are genomically abnormal, showing a specific genetic profile for the 5q- syndrome. *Leukemia* **23**, 664-672 (2009).
20. Medyouf, H.*, et al.* Myelodysplastic cells in patients reprogram mesenchymal stromal cells to establish a transplantable stem cell niche disease unit. *Cell stem cell* **14**, 824-837 (2014).

21. Ribezzo, F*., et al.* Rps14, Csnk1a1 and miRNA145/miRNA146a deficiency cooperate in the clinical phenotype and activation of the innate immune system in the 5q- syndrome. *Leukemia* (2019).
22. Chasis, J.A. & Mohandas, N. Erythroblastic islands: niches for erythropoiesis. *Blood* **112**, 470-478 (2008).
23. Crane, G.M., Jeffery, E. & Morrison, S.J. Adult haematopoietic stem cell niches. *Nature reviews. Immunology* **17**, 573-590 (2017).
24. Chen, X*., et al.* Induction of myelodysplasia by myeloid-derived suppressor cells. *The Journal of clinical investigation* **123**, 4595-4611 (2013).
25. Dong, L*., et al.* Leukaemogenic effects of Ptpn11 activating mutations in the stem cell microenvironment. *Nature* **539**, 304-308 (2016).
26. Sternlicht, M.D*., et al.* The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell* **98**, 137-146 (1999).
27. Radisky, D.C*., et al.* Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature* **436**, 123-127 (2005).
28. Xia, J*., et al.* Somatic mutations and clonal hematopoiesis in congenital neutropenia. *Blood* **131**, 408-416 (2018).
29. Sperling, A.S., Gibson, C.J. & Ebert, B.L. The genetics of myelodysplastic syndrome: from clonal haematopoiesis to secondary leukaemia. *Nature reviews. Cancer* **17**, 5-19 (2017).
30. Shlush, L.I*., et al.* Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature* **506**, 328-333 (2014).
31. Jan, M*., et al.* Clonal evolution of preleukemic hematopoietic stem cells precedes human acute myeloid leukemia. *Science translational medicine* **4**, 149ra118 (2012).
32. Meisel, M*., et al.* Microbial signals drive pre-leukaemic myeloproliferation in a Tet2-deficient host. *Nature* **557**, 580-584 (2018).
33. Mirantes, C., Passegue, E. & Pietras, E.M. Pro-inflammatory cytokines: emerging players regulating HSC function in normal and diseased hematopoiesis. *Experimental cell research* **329**, 248-254 (2014).
34. Cai, Z*., et al.* Inhibition of Inflammatory Signaling in Tet2 Mutant Preleukemic Cells Mitigates Stress-Induced Abnormalities and Clonal Hematopoiesis. *Cell stem cell* **23**, 833-849 e835 (2018).
35. Hunter, C.A. & Jones, S.A. IL-6 as a keystone cytokine in health and disease. *Nature immunology* **16**, 448-457 (2015).
36. Tourlakis, M.E*., et al.* Deficiency of Sbds in the mouse pancreas leads to features of Shwachman-Diamond syndrome, with loss of zymogen granules. *Gastroenterology* **143**, 481-492 (2012).
37. Hanahan, D. & Weinberg, R.A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).
38. Hayden, M.S. & Ghosh, S. Shared principles in NF-kappaB signaling. *Cell* **132**, 344-362 (2008).
39. Zhang, Q., Lenardo, M.J. & Baltimore, D. 30 Years of NF-kappaB: A Blossoming of Relevance to Human Pathobiology. *Cell* **168**, 37-57 (2017).
40. Nakagawa, M.M. & Rathinam, C.V. Constitutive Activation of the Canonical NF-kappaB Pathway Leads to Bone Marrow Failure and Induction of Erythroid Signature in Hematopoietic Stem Cells. *Cell reports* **25**, 2094-2109 e2094 (2018).
41. Ben-Neriah, Y. Regulatory functions of ubiquitination in the immune system. *Nature immunology* **3**, 20-26 (2002).
42. Hayden, M.S. & Ghosh, S. NF-kappaB, the first quarter-century: remarkable progress and outstanding questions. *Genes & development* **26**, 203-234 (2012).
43. Mercurio, F*., et al.* IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* **278**, 860-866 (1997).

44. Schepers, K.*, et al.* Myeloproliferative neoplasia remodels the endosteal bone marrow niche into a self-reinforcing leukemic niche. *Cell stem cell* **13**, 285-299 (2013).
45. Georgakilas, A.G., Martin, O.A. & Bonner, W.M. p21: A Two-Faced Genome Guardian. *Trends in molecular medicine* **23**, 310-319 (2017).
46. Coppe, J.P., Desprez, P.Y., Krtolica, A. & Campisi, J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annual review of pathology* **5**, 99-118 (2010).
47. Abdul-Aziz, A.M.*, et al.* Acute myeloid leukemia induces protumoral p16INK4a-driven senescence in the bone marrow microenvironment. *Blood* **133**, 446-456 (2019).
48. Chien, Y.*, et al.* Control of the senescence-associated secretory phenotype by NF-kappaB promotes senescence and enhances chemosensitivity. *Genes & development* **25**, 2125-2136 (2011).
49. Rovillain, E.*, et al.* Activation of nuclear factor-kappa B signalling promotes cellular senescence. *Oncogene* **30**, 2356-2366 (2011).
50. Geyh, S.*, et al.* Insufficient stromal support in MDS results from molecular and functional deficits of mesenchymal stromal cells. *Leukemia* **27**, 1841-1851 (2013).
51. Zhao, Y.*, et al.* Down-regulation of Dicer1 promotes cellular senescence and decreases the differentiation and stem cell-supporting capacities of mesenchymal stromal cells in patients with myelodysplastic syndrome. *Haematologica* **100**, 194-204 (2015).
52. Alter, B.P. Diagnosis, genetics, and management of inherited bone marrow failure syndromes. *Hematology. American Society of Hematology. Education Program*, 29-39 (2007).
53. Donadieu, J.*, et al.* Classification of and risk factors for hematologic complications in a French national cohort of 102 patients with Shwachman-Diamond syndrome. *Haematologica* **97**, 1312-1319 (2012).
54. Grinenko, T.*, et al.* Hematopoietic stem cells can differentiate into restricted myeloid progenitors before cell division in mice. *Nature communications* **9**, 1898 (2018).
55. Zhao, J.L.*, et al.* Conversion of danger signals into cytokine signals by hematopoietic stem and progenitor cells for regulation of stress-induced hematopoiesis. *Cell stem cell* **14**, 445-459 (2014).
56. Chen, X.*, et al.* Bone Marrow Myeloid Cells Regulate Myeloid-Biased Hematopoietic Stem Cells via a Histamine-Dependent Feedback Loop. *Cell stem cell* **21**, 747-760 e747 (2017).
57. Mossadegh-Keller, N.*, et al.* M-CSF instructs myeloid lineage fate in single haematopoietic stem cells. *Nature* **497**, 239-243 (2013).
58. Casanova-Acebes, M.*, et al.* Rhythmic modulation of the hematopoietic niche through neutrophil clearance. *Cell* **153**, 1025-1035 (2013).
59. Sturge, C.R.*, et al.* TLR-independent neutrophil-derived IFN-gamma is important for host resistance to intracellular pathogens. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 10711-10716 (2013).
60. Tecchio, C., Micheletti, A. & Cassatella, M.A. Neutrophil-derived cytokines: facts beyond expression. *Frontiers in immunology* **5**, 508 (2014).
61. Essers, M.A.*, et al.* IFNalpha activates dormant haematopoietic stem cells in vivo. *Nature* **458**, 904-908 (2009).
62. Baldridge, M.T., King, K.Y., Boles, N.C., Weksberg, D.C. & Goodell, M.A. Quiescent haematopoietic stem cells are activated by IFN-gamma in response to chronic infection. *Nature* **465**, 793-797 (2010).
63. Pietras, E.M.*, et al.* Re-entry into quiescence protects hematopoietic stem cells from the killing effect of chronic exposure to type I interferons. *The Journal of experimental medicine* **211**, 245-262 (2014).
64. Boehm, U.*, et al.* Two families of GTPases dominate the complex cellular response to IFN-gamma. *J Immunol* **161**, 6715-6723 (1998).

65. Beerman, I.*, et al.* Functionally distinct hematopoietic stem cells modulate hematopoietic lineage potential during aging by a mechanism of clonal expansion. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 5465-5470 (2010).

66. Swindell, W.R.*, et al.* Robust shifts in S100a9 expression with aging: a novel mechanism for chronic inflammation. *Scientific reports* **3**, 1215 (2013).

67. Bresnick, A.R., Weber, D.J. & Zimmer, D.B. S100 proteins in cancer. *Nature reviews. Cancer* **15**, 96-109 (2015).

68. Benarafa, C.*, et al.* SerpinB1 protects the mature neutrophil reserve in the bone marrow. *Journal of leukocyte biology* **90**, 21-29 (2011).

69. Parsa, R.*, et al.* BAFF-secreting neutrophils drive plasma cell responses during emergency granulopoiesis. *The Journal of experimental medicine* **213**, 1537-1553 (2016).

70. Armstrong, A.J.*, et al.* Long-term survival and biomarker correlates of tasquinimod efficacy in a multicenter randomized study of men with minimally symptomatic metastatic castration-resistant prostate cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* **19**, 6891-6901 (2013).

71. Yan, L.*, et al.* Beneficial effects of quinoline-3-carboxamide (ABR-215757) on atherosclerotic plaque morphology in S100A12 transgenic ApoE null mice. *Atherosclerosis* **228**, 69-79 (2013).

72. Helmersson, S., Sundstedt, A., Deronic, A., Leanderson, T. & Ivars, F. Amelioration of experimental autoimmune encephalomyelitis by the quinoline-3-carboxamide paquinimod: reduced priming of proinflammatory effector CD4(+) T cells. *The American journal of pathology* **182**, 1671-1680 (2013).

A

ADDENDUM

LIST OF ABBREVIATIONS

AGM	Aorta-gonad-mesonephros
AML	Acute myeloid leukemia
ANG	Angiogenin
ASXL1	Additional sex combs like 1
AZA	Azacitidine
BF	Bone fraction
BM	Bone marrow
BMF	Bone marrow failure
CAR	CXCL12-abundant reticular
CCL3	C-C Motif chemokine ligand 3
CEBPA	CCAAT/enhancer binding protein alpha
CFU-C	Colony forming unit culture
CFU-F	Fibroblast colony forming unit
CHIP	Clonal hematopoiesis of indeterminate potential
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CXCL4	Chemokine (C-X-C motif) ligand 4/ Platelet factor 4
CXCR4	C-X-C motif chemokine receptor 4
c-KIT	Mast/stem cell growth factor receptor / CD117
DAMP	Damage-associated molecular pattern
DBA	Diamond-Blackfan anemia
DC	Dyskeratosis Congenita
DDR	DNA damage response
DHE	Dihydroethidium
DKK1	Dickkopf-1
DNMT3A	DNA methyltransferase 3
DTA	Diphtheria toxin A
ECM	Extracellular matrix
EIF6	Eukaryotic translation initiation factor 6
ES	Enrichment score
EYFP	Enhanced yellow fluorescent protein
FC	Fold change
FDR	False discovery rate
FGF	Fibroblast growth factor
FPKM	Fragments per kilobase of exon per million fragments mapped
G-CSF	Granulocyte colony-stimulating factor
G-CSFR	G-CSF receptor

GFI-1	Growth factor independent-1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte-macrophage progenitor
γH2AX	Ser139-phosphorylated H2AX histone
GSEA	Gene set enrichment analysis
HDC	Histidine decarboxylase
HGB	Hemoglobin
HPC	Hematopoietic progenitor cell
HR-MDS	High-risk myelodysplastic syndromes
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IFN	Interferon
Iigp1	Interferon inducible GTPase 1
IκB	Inhibitor of κB
IKK	IκB kinase
IL-1β	Interleukin 1 Beta
IPSS	International prognostic scoring system
IT-HSC	Intermediate-term HSC
LEPR	Leptin receptor
LMPP	lymphoid-primed multipotent progenitor
LPS	Lipopolysaccharide
LR-MDS	Low-risk myelodysplastic syndromes
LR-PSS	Lower-risk prognostic scoring system
LSC	Leukemic stem cells
LT-HSC	Long-term HSC
M-CSF	Macrophages colony stimulating factors
M-CSFR	M-CSF receptor
MDS	Myelodysplastic syndromes
MDSC	Myeloid-derived suppressor cell
MEP	Megakaryocyte erythroid progenitor
MFI	Mean fluorescence intensity
Mks	Megakaryocytes
MLP	Multi-lymphoid progenitor
MMP	Matrix metallopeptidase
MPC	Mesenchymal progenitor cell
MPD	Myeloproliferative disease
MPL	Myeloproliferative leukemia protein / Thrombopoietin receptor
MPN	Myeloproliferative neoplasms
MPO	Myeloperoxidase

MPP	Multipotent progenitor
MSC	Mesenchymal stem/stromal cell
MSigDB	Molecular signature database
NES	Normalized enrichment score
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor binding near the κ light-chain gene in B cells
NFKBIA	NF-κB inhibitor alpha
OCS	*Osterix*-cre *Sbds*-flox strain
PB	Peripheral blood
PCA	Principal component analysis
PLT	Platelet
PRX1	Paired related homeobox 1
PTPN11	Protein tyrosine phosphatase, non-receptor type 11
RAGE	Receptor for advanced glycation end products
RBC	Red blood cell
ROS	Reactive oxygen species
S100A8	S100 calcium binding protein A8
S100A9	S100 calcium binding protein A9
SASP	Senescence-associated secretory profile
SBDS	Shwachman-Bodian-Diamond Syndrome gene
Sca-1	Stem cell antigen 1
SCF	Stem cell factor
SCN	Severe congenital neutropenia
SDF1	Stromal cell-derived factor 1
SDS	Shwachman-Diamond syndrome
SEM	Standard error of the mean
SLAM	Signaling lymphocyte activation molecule
ST-HSC	Short-term HSC
TET2	Tet Methylcytosine Dioxygenase 2
TGF-β	Transforming Growth Factor Beta
TLR	Toll-like receptor
TMRM	Tetramethylrhodamine, methyl ester
TPO	Thrombopoietin
WBC	White blood cell

ENGLISH SUMMARY

Mature blood cells are vital to mammalians for oxygen transportation, protection against pathogens, and wound healing. Most mature blood cells are short-lived and require life-long replenishment through hematopoiesis. Hematopoietic stem cells (HSCs) are pluripotent cells defined by their capability to duplicate themselves (self-renewal), and to generate blood cells of all the lineages (differentiation). In adulthood, HSCs reside predominantly in the bone marrow and are maintained at a metabolically inactive state (quiescent) during homeostasis. Upon the demand for hematopoiesis, HSCs gradually lose pluripotency to generate progenitor cells, which are highly proliferative and give rise to terminally differentiated blood cells.

The properties of HSCs including quiescence, proliferation and differentiation are maintained by both intrinsic programs from HSCs and extrinsic signals from the bone marrow microenvironment, also known as the HSC niche. Multiple cellular components of the HSC niche have been identified, including stem cells and progenitor cells of the mesenchyme. These mesenchymal cells localize in the vicinity of HSCs and regulate them via direct cell-cell contact or secretion of soluble factors.

When the delicate balance of HSC regulation is tilted, hematological clonal disorders can arise, including myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML). MDS are characterized by ineffective hematopoiesis and the propensity for the development of AML. AML is a form of cancer characterized by uncontrolled proliferation and accumulation of immature myeloid cells in the blood and the bone marrow. Numerous of studies have focused on identifying the HSC-intrinsic events leading to leukemia, while the contribution of the HSC niche to leukemogenesis is less well defined. Our group have earlier demonstrated in a genetic mouse model that perturbation of the HSC niche can disrupt normal hematopoiesis in ways reminiscent of human MDS, including the development of AML, thus providing the first proof-of-principle for niche induced oncogenesis in the hematopoietic system. However, the molecular mechanism underlying the niche-driven leukemogenesis remain largely unknown. In this dissertation, we aim to further address these key issues and provide answers to the following research questions:
1. What are the molecular events underlying the HSC niche-driven clonal transformation of hematopoiesis?
2. What is the relevance of these molecular events to human diseases?

We approached these questions by interrogating the niche and HSC interaction, both in genetic mouse models and patient samples of bone marrow failure (BMF) syndromes. MDS and Shwachman-Diamond syndrome (SDS) provide excellent disease models to address our

research questions. SDS is an inherited BMF syndrome caused by monogenic mutations in the Shwachman-Bodian-Diamond syndrome (*SBDS*) gene resulting in both blood and bone phenotypes in the patients.

To provide mechanistic insights underlying the concept of niche induced oncogenesis, in chapter 2 we embarked on a mouse model with targeted deletion of *Sbds* in *Osterix*-expressing mesenchymal progenitor cells (MPCs). Osteoporosis and myelodysplasia were observed in mutant mice, faithfully recapitulating the skeletal and hematopoietic phenotypes in SDS patients. Disruption of MPCs led to genotoxic stress in the hematopoietic stem and progenitor cells (HSPCs), characterized by mitochondrial dysfunction, accumulation of reactive oxygen species (ROS), DNA damage, and consequently cell cycle checkpoint activation. Our data revealed the activation of p53 pathway in *Sbds*-deficient MPCs, which is commonly associated with the pathogenesis of ribosomopathies. Targeted deletion of *Trp53* in *Sbds*-deficient MPCs rescued the osteoporotic and the genotoxic stress phenotypes, confirming that the phenotypes are p53 dependent. Next, we aimed to identify human disease relevant niche factors downstream of p53 driving genotoxic stress in HSPCs. We performed transcriptome profiling in MPCs isolated from the mouse model as well as the patients of BMF syndromes including SDS, MDS and Diamond-Blackfan anemia (DBA). Significant upregulation of genes encoding inflammatory molecules were found in mutant mice and patients suffering from BMF syndromes associated with high propensity for leukemia evolution (SDS and MDS), including *S100A8* and *S100A9*, which are *bona fide* p53 downstream targets. Overexpression of S100a8 and S100a9 proteins was confirmed in the *Sbds*-deficient MPCs, associated with increased secretion of S100a8/9 heterodimer in the plasma of the mutant mice. S100a8/9 induced genotoxic stress in HSPCs both in *ex vivo* and *in vivo* settings and signaled through their canonical receptor toll-like receptor 4 (TLR4); administration of TLR4 antibody to mutant mice partially rescued the phenotypes. Transplantation of wildtype HSPCs into S100a9 transgenic mice demonstrated that niche-derived S100a8/9 is sufficient to drive genotoxic stress in HSPCs. Finally, to expand our findings into broader relevance of human disease, we focused on MDS, the principle leukemia predisposition syndrome. Transcriptome profiling of the MPCs purified from low risk (LR)-MDS patients revealed significant upregulation of *S100A8* and *S100A9*, associated with the activation of p53 and TLR4 pathways. These patients can be categorized into S100 niche positive and S100 niche negative groups based on the mesenchymal expression of S100A8 and S100A9, which are independent of the current prognostic factors. The S100 niche positive patients demonstrated a worse disease outcome, characterized by increased leukemic evolution and decreased progression-free survival.

Collectively, we identified a novel mechanistic insight that the mesenchymal niche can drive genotoxic stress in heterotypic HSPCs through p53-S100A8/9-TLR4 inflammatory signaling,

and that the mesenchymal expression of S100A8/9 can predict leukemic evolution in LR-MDS patients. Notably, S100A8 and S100A9 were among many other inflammatory molecules upregulated in the MPCs of LR-MDS patients; an important question emerged from these findings was what drove the inflammatory signaling?

In chapter 3, we aimed to identify the upstream regulator of mesenchymal inflammation in LR-MDS. Transcriptome profiling of MPCs isolated from LR-MDS patients revealed enriched gene signatures in NF-κB pathway and upregulated expression of NF-κB inhibitor alpha (*NFKBIA*) in comparison to healthy controls, indicating mesenchymal activation of NF-κB pathway is common in LR-MDS. The expression of *NFKBIA* significantly correlated with the expression of inflammatory molecules, including *bona fide* NF-κB downstream targets. The activation of NF-κB pathway in LR-MDS mesenchyme was further supported by the increased phosphorylation of p65 (p-p65). To investigate the consequence of mesenchymal NF-κB activation on HSPCs, we developed an *ex vivo* model with OP9 stromal cells by overexpressing a constitutively active form of IKK2, which is a kinase upstream regulator of NF-κB. The activation of NF-κB signaling in OP9 cells was verified by upregulated p-p65 and increased expression levels of inflammatory molecules which are downstream targets of NF-κB. Coculture of healthy bone marrow cells with OP9 cells harboring activated NF-κB signaling resulted in attenuation of the number and function of HSPCs. Altogether, we provided experimental evidence that mesenchymal NF-κB activation is an upstream driver of niche inflammation and contributes to BMF in LR-MDS.

In chapter 4, we focused on identifying the contribution of cytopenia in the pathogenesis and leukemic evolution of BMF syndromes. Neutropenia, defined by the loss of mature neutrophils in peripheral blood, is a hallmark of SDS and other congenital neutropenia syndromes with predisposition to AML. We exploited a mouse model of neutropenia established previously in our group, by transplanting fetal liver hematopoietic cells carrying targeted deletion of *Sbds* in *Cebpa*-expressing cells or their control counterparts into wildtype recipients. Recipients of the mutant cells developed profound and sustained neutropenia, while their HSCs remained genetically *Sbds* proficient. HSCs in neutropenia remained quiescent, as suggested by unaltered numbers and frequencies in comparison to their control counterparts, and was confirmed by cell cycle analysis. Lack of replicative stress was further suggested by unaltered levels of DNA damage. Consistently, transcriptome profiling revealed enriched gene signatures of a glycolytic metabolic state in HSCs exposed to neutropenia with reduced levels of mitochondria-derived ROS. In contrast, multipotent progenitors (MPPs) in neutropenia demonstrated decreased numbers and increased cycling, in line with the enriched gene signatures of an active metabolic state. In addition, both HSCs and MPPs exposed to neutropenia demonstrated enriched transcriptional programs in myeloid commitment, suggesting that HSCs give rise to transiently proliferating MPPs via division

uncoupled differentiation to compensate for the loss of mature neutrophils. Interestingly, HSCs in neutropenia displayed amelioration of typically ageing-associated phenotypes, such as expansion of immunophenotypically defined HSCs and loss of serial repopulating ability. Mechanistically, interferon (IFN) signaling, a known physiologic driver of stem cell exhaustion, was mitigated in HSCs exposed to neutropenia, suggesting that neutrophils may be negative regulators of hematopoiesis through the induction of IFN/inflammatory signaling. In addition, remodeling of the HSC niche was observed upon prolonged exposed to neutropenia, as defined by the alterations in frequencies and transcriptional landscapes of mesenchymal stromal cells and endothelial cells. Altogether, our data demonstrated that HSCs are preserved in chronic neutropenia; the precise molecular mechanisms driving HSC preservation are currently under investigation.

In conclusion, the research performed in this dissertation has provided novel mechanistic insights into the contribution of the HSC niche in the pathogenesis of BMF syndromes. Our findings suggest that defects in hematopoietic cells and the bone marrow microenvironment may work synergistically to induce BMF and predisposition for clonal transformation, and that inflammatory signaling is an important underlying mechanism. The shortage of blood cells in neutropenia and the demand this poses on HSCs were shown not to lead to replicative stress, exhaustion, stem cell failure or contribute to transformation. These findings pave the way for further research in understanding the molecular and cellular interplay between diverse bone marrow components in stem cell failure and malignant transformation. This is anticipated to lead to novel (niche-instructed) prognostic factors in LR-MDS, and might instruct prime the development of novel therapeutics to target the HSC niche in BMF syndromes and leukemogenesis.

NEDERLANDSE SAMENVATTING

Rijpe bloedcellen zijn van vitaal belang voor zoogdieren in verband met zuurstoftransport, bescherming tegen pathogenen en wondgenezing. De meeste bloedcellen hebben een korte levensduur waardoor continue aanvulling, door middel van hematopoëse in het beenmerg, vereist wordt gedurende heel het leven. Hematopoëtische stamcellen (HSCs) zijn pluripotente cellen die gedefinieerd worden door hun vermogen om zichzelf te vernieuwen en bloedcellen van elke soort te genereren (differentiatie). Op volwassen leeftijd bevinden HSCs zich hoofdzakelijk in het beenmerg en zijn ze, gedurende homeostase, grotendeels metabool inactief (in 'sluimerstand'). Wanneer de vraag naar bloedcellen opeens stijgt (bv. bij infectie), worden de HSCs geactiveerd en verliezen ze geleidelijk hun pluripotente eigenschappen zodat ze vorm kunnen geven aan voorlopercellen die op hun beurt zeer proliferatief zijn en aan de vraag van extra bloedcellen kunnen voldoen.

De verschillende eigenschappen van een HSC (sluimerstand, proliferatie en/of differentiatie) worden gedirigeerd door zowel intrinsieke programma's van HSCs als extrinsieke signalen vanuit de beenmergomgeving, ook bekend als de HSC-niche. Meerdere cellulaire componenten van de HSC-niche zijn reeds geïdentificeerd, waaronder mesenchymale stamcellen en voorlopercellen. Deze mesenchymale cellen lokaliseren zich in de nabijheid van HSCs en reguleren deze waarschijnlijk via direct cel-cel contact of door uitscheiding van specifieke factoren.

Wanneer de delicate balans van HSC-regulatie wordt verstoord, kunnen hematologische klonale stoornissen optreden, waaronder het myelodysplastisch syndroom (MDS) en acute myeloïde leukemie (AML). MDS wordt gekenmerkt door ineffectieve hematopoëse en een vergroot risico op het ontwikkelen van AML. AML is een vorm van kanker die wordt gekenmerkt door ongecontroleerde proliferatie en accumulatie van niet-rijpe myeloïde cellen in het bloed en beenmerg. Tal van onderzoeken hebben zich gericht op het identificeren van de HSC-intrinsieke gebeurtenissen die leiden tot leukemie, terwijl de bijdrage van de HSC-niche hierin minder goed is bestudeerd. Onze groep heeft eerder aangetoond dat verstoring van de HSC-niche, in een genetisch muis model, de normale hematopoëse kan verstoren op een manier die doet denken aan MDS, inclusief het ontwikkelen van AML. Dit ondersteunde het idee dat de HSC-niche verantwoordelijk kan zijn voor het ontwikkelen van een maligniteit in het bloed. De moleculaire mechanismen die ten grondslag ligt aan de 'niche-gedreven leukemogenese' blijven echter grotendeels onbekend. In dit proefschrift proberen we deze belangrijke kwesties verder uit te diepen en antwoord te geven op de volgende onderzoeksvragen:

1. Wat zijn de moleculaire gebeurtenissen die ten grondslag liggen aan de HSC niche-gestuurde klonale transformatie van hematopoëse?
2. Wat is de relevantie van deze moleculaire gebeurtenissen voor menselijke ziekten?

We hebben deze vragen trachten te beantwoorden door de niche- en HSC-interactie te onderzoeken in zowel genetische muismodellen als in patiënten met beenmergfalen (BMF). MDS en Shwachman-Diamond-syndroom (SDS) zijn hierin uitstekende ziektemodellen om onze onderzoeksvragen te adresseren. SDS is een congenitaal BMF-syndroom veroorzaakt door mutaties in het Shwachman-Bodian-Diamond-syndroom (*SBDS*) -gen, resulterend in zowel een bloed- als een botfenotype.

Om mechanistische inzichten te krijgen in het concept van niche-geïnduceerde oncogenese, hebben we in hoofdstuk 2 een muismodel onderzocht met gerichte deletie van het *Sbds* gen in *Osterix*-producerende mesenchymale voorlopercellen (MPCs). Osteoporose en myelodysplasie werden waargenomen in mutante muizen, een mooie weerspiegeling van de bot- en hematopoëtische fenotypen die we kennen in SDS-patiënten. Verstoring van MPCs leidde tot genotoxische stress in de hematopoëtische stam- en voorlopercellen (HSPCs), gekenmerkt door mitochondriale disfunctie, accumulatie van zuurstofradicalen (ROS), DNA-beschadiging en dientengevolge activatie van controlepunten in de celcyclus. Tevens onthulden onze data activatie van de p53-pathway in *Sbds*-deficiënte MPCs; hetgeen geassocieerd wordt met de pathogenese van ribosomopathieën als SDS. Vervolgens kon gerichte deletie van *Trp53* in *Sbds*-deficiënte MPCs de osteoporotische en genotoxische fenotypen redden, wat bevestigt dat de fenotypen afhankelijk zijn van p53. Vervolgens hebben we ernaar gestreefd relevante nichefactoren binnen de p53 pathway te identificeren die van belang zouden kunnen zijn in ziekte bij de mens. Hiervoor keken we gedetailleerd naar zowel het transcriptoom van MPCs geïsoleerd uit het muismodel als uit patiënten met de BMF-syndromen SDS, MDS en Diamond-Blackfan-anemie (DBA). Significante upregulatie van genen die coderen voor ontstekingsmoleculen (vb. *S100A8* en *S100A9*) werd gevonden in mutante muizen en specifieke BMF-patiënten met een verhoogde kans op het ontwikkelen van leukemie (SDS en MDS). Overproductie van S100a8- en S100a9-eiwit werd bevestigd in de *Sbds*-deficiënte MPCs, wat geassocieerd was met verhoogde uitscheiding van het S100a8/9-heterodimeer in plasma van de mutante muizen. Zowel in *ex vivo* als *in vivo* experimenten induceerde S100a8/9 genotoxische stress in HSPCs via de toll-like receptor 4 (TLR4); toediening van TLR4-antilichamen aan mutante muizen redde de fenotypen gedeeltelijk. Transplantatie van wildtype HSPCs in S100a9-transgene muizen toonde aan dat van niche afkomstige S100a8/9 voldoende was om genotoxische stress in HSPCs te veroorzaken. Om onze bevindingen verder uit te breiden naar een bredere toepassing op menselijke ziekten, hebben we ons tot slot gericht op MDS, het meest voorkomende leukemie predispositie syndroom. Transcriptoom onderzoek van de MPCs afkomstig van laag risico (LR)-MDS-patiënten onthulde een significante upregulatie van *S100A8* en *S100A9*. Deze groep van patiënten kon worden ingedeeld in een S100-hoog-niche groep en een S100-laag-niche groep op basis van de expressie van S100A8 en S100A9 in het mesenchym. De S100-hoog-niche patiënten vertoonden een slechtere uitkomst van de ziekte, gekenmerkt door een verhoogde kans op het ontwikkelen van leukemie, weerspiegeld in een verkorte progressievrije overleving.

Concluderend leidde dit onderzoek tot een nieuw concept van 'niche geïnduceerde genotoxische stress' in HSCs en mechanistische inzichten hierin, met name activatie van signalen afkomstig uit een p53-S100A8/9-TLR4 as. Activatie van deze mesenchymale-HSC signaalas was geassocieerd met de evolutie naar AML in LR-MDS-patiënten. Opmerkelijk was dat S100A8 en S100A9 slechts twee componenten waren van vele andere ontstekingsmoleculen die verhoogd tot expressie kwamen in de MPCs van LR-MDS-patiënten. Een belangrijke vraag die naar voren kwam uit deze bevindingen was: wat is de onderliggende oorzaak van al die ontstekingssignalen?

Het onderzoek beschreven in hoofdstuk 3 had tot doel een mogelijke overkoepelende oorzaak voor de 'globale' mesenchymale ontsteking in LR-MDS te identificeren. Transcriptoom-profilering van MPCs afkomstig van LR-MDS-patiënten onthulde activatie van het NF-κB-signaalpad (waaronder verhoogde expressie van NF-κB-remmer alpha (*NFKBIA*), in vergelijking met gezonde mensen. Dit gaf aan dat mesenchymale activering van NF-κB-pathway veel voorkomt in LR-MDS. Activatie van het NF-κB-signaalpad in LR-MDS-mesenchym werd verder ondersteund door de verhoogde fosforylering van p65 (p-p65). Om de consequenties van mesenchymale NF-κB-activatie op HSPCs te onderzoeken, ontwikkelden we een *ex vivo* model met stromale cellen waarin IKK2 geactiveerd is, hetgeen leidt tot upregulatie van NF-κB. Wanneer we de IKK2 producerende stromale cellen samen kweekten met gezonde beenmergcellen leidde dit tot vermindering van het aantal HSPCs en hun functie. Samenvattend laten onze resultaten zien dat activatie van het mesenchymale NF-κB signaalpad een veel voorkomende oorzaak van niche ontsteking is in LR-MDS en bijdraagt aan BMF in deze ziekte.

In hoofdstuk 4 richten we ons op de bijdrage van cytopenie aan de pathogenese van leukemie in BMF-syndromen. Neutropenie, gedefinieerd door de afwezigheid van rijpe neutrofielen in perifeer bloed, is een kenmerk van SDS en andere aangeboren neutropeniesyndromen met een verhoogde kans op het ontwikkelen van AML. We gebruikten een neutropeen muismodel waarbij we foetale hematopoëtische cellen, met een gerichte deletie van het *Sbds* gen in *Cebpa* producerende cellen, transplanteerden in wild type muizen. Ontvangers van de mutante cellen ontwikkelden een diepe en aanhoudende neutropenie; dit terwijl de HSCs genetisch geen afwijkingen hebben. Neutropenie leidde niet tot activatie of verhoogde celdeling in HSCs. Dit werd ondersteund door de bevinding dat de HSCs geen celdelingsstress of DNA-schade vertoonden. Genexpressie-analyse suggereerde dat de HSCs afkomstig van neutropene muizen in een glycolytische metabole toestand verkeerden, wat in overeenstemming lijkt met het aantonen van een verminderd niveau van mitochondriale ROS. Daarentegen waren multipotente voorlopercellen (MPPs) gedurende neutropenie afgenomen in aantal en hadden ze een verhoogde celcyclus. Dit kwam overeen met transcriptionele veranderingen die wezen op een actieve metabole toestand. Zowel HSCs

als MPPs vertoonden een verschuiving naar myeloïde differentiatie. De data suggereren dat neutropenie met name opgevangen wordt door activatie en celdeling van vroege voorlopercellen, terwijl HSCs in ruste blijven en niet onder stress komen te staan. Sterker nog, HSCs in neutropenie vertoonden een verbetering van typisch ouderdom-geassocieerde kenmerken zoals expansie en verlies van functie. Mechanistisch gezien was interferon (IFN) -signalering, een bekende fysiologische aandrijver van stamceluitputting, verlaagd in HSCs die waren blootgesteld aan neutropenie. Dit zou kunnen suggereren dat neutrofielen een op lange termijn nadelig effect hebben op de functie van HSCs en bijdragen aan hun veroudering door de inductie van IFN-signalering. Vertraagde veroudering van stamcellen bij neutropenie ging gepaard met veranderingen in de samenstelling en moleculaire opmaak van de HSC-niche. Al met al hebben onze gegevens aangetoond dat HSCs functioneel worden behouden bij chronische neutropenie; de precieze moleculaire mechanismen die zorgen voor HSC-preservatie worden momenteel onderzocht.

Concluderend heeft het onderzoek dat in dit proefschrift is uitgevoerd nieuwe mechanistische inzichten opgeleverd omtrent de bijdrage van de HSC-niche aan de pathogenese van BMF-syndromen. Onze bevindingen suggereren dat defecten in de beenmergomgeving bijdragen aan BMF en klonale evolutie en dat ontstekingssignalering een belangrijk onderliggend mechanisme is. Het tekort aan bloedcellen in neutropenie, en de druk die dat legt op HSCs, bleek niet bij te dragen aan delingsstress, uitputting, stamcel-falen of klonale transformatie. Deze bevindingen banen de weg voor verder onderzoek naar de moleculaire en cellulaire wisselwerking tussen verschillende beenmergcomponenten bij stamcel-falen en maligne transformatie. Dit zal naar verwachting leiden tot nieuwe (niche-geïnstrueerde) prognostische factoren in LR-MDS en de ontwikkeling van nieuwe therapieën die gericht zijn op de HSC-niche in BMF-syndromen en leukemogenese.

中文提要

成熟的血细胞在哺乳动物机体内有至关重要的生理功能，包括运送氧气，抵抗病原体和促进伤口愈合。大多数成熟的血细胞生命短暂，终生需要通过造血作用来补充。造血干细胞（HSC）由于具有复制自己（自我更新），和生成所有谱系血细胞（多向分化）的潜能而被定义为多能细胞。在成年期，HSC主要存在于骨髓中，在稳态时处于不活跃的代谢状态（静息）。当机体有造血需求时，HSC逐步失去多能性从而生成高度增殖的造血祖细胞，并最终分化为成熟的血细胞。

HSC的状态，包括静息，增殖和分化，是由HSC的内在程序和骨髓微环境（龛位）的外在信号共同维持的。多种HSC龛位的细胞组分已经鉴定，包括间充质干细胞和祖细胞。这些间充质细胞分布在HSC附近，通过直接的细胞间接触或分泌可溶性因子来调节HSC。

打破调节HSC的微妙平衡可能会导致克隆性血液系统疾病，包括骨髓增生异常综合征（MDS）和急性髓系白血病（AML）。 MDS的临床表现为造血功能丧失和向AML转化的倾向。 AML是一种癌症，表现为未成熟的髓细胞在血液和骨髓中不受控制的增殖和累积。之前许多研究都集中在鉴定导致白血病的HSC内在事件，而对HSC龛位与白血病发生联系的研究相对欠缺。我们研究组早先在一个遗传小鼠模型中证明，对HSC龛位的干扰会破坏正常的造血功能，导致与人类MDS类似的症状，包括向AML的转化，从而为造血系统中龛位诱导的肿瘤形成提供了第一个原理验证。然而，龛位诱导白血病发生的分子机理尚未明确。在本论文中，我们致力于进一步探讨这些重要事件，并为下列研究问题提供答案：
一.HSC龛位诱导造血系统肿瘤形成的分子机理是什么?
二.这些分子机理与人类疾病的相关性是什么?

我们通过研究小鼠模型和骨髓衰竭（BMF）综合征患者样本中的HSC与其龛位的相互作用来解决上述问题。MDS和Shwachman-Diamond综合征（SDS）为研究这些问题提供了适宜的疾病模型。 SDS是由Shwachman-Bodian-Diamond综合征基因（SBDS）单基因突变引起的遗传性BMF综合征，导致患者血液和骨骼的病变。

在第二章中，我们运用在表达成骨细胞特异性转录因子（Osterix）的间充质祖细胞（MPC）中特异性Sbds剔除的小鼠模型，来研究龛位诱导肿瘤发生的分子机理。基因剔除小鼠呈现了骨质疏松症和骨髓增生异常的表型，准确的模拟了SDS患者的骨骼和血液系统的症状。干扰MPC导致造血干细胞和祖细胞（HSPC）的应激基因毒性反应，表现为线粒体功能障碍，活性氧（ROS）累积，DNA损伤，并引发细胞周期检测点激活。我们的数据揭示了Sbds缺陷型小鼠MPC中p53信号通路的活化，这通常与

核糖体疾病的发病机制有关。在Sbds缺陷型小鼠MPC中特异性剔除Trp53拯救了骨质疏松和应激基因毒性的表型，证实了该表型是依赖于p53的。我们下一步的研究致力于确定在HSPC中p53下游诱导应激基因毒性反应的，与人类疾病相关的龛位因子。我们对从小鼠模型中以及BMF综合征患者中分离的MPC进行了转录组分析，包括SDS，MDS和Diamond-Blackfan贫血症（DBA）患者。我们在基因剔除小鼠以及具有高倾向转化白血病的BMF综合征（SDS和MDS）患者中发现了炎性分子基因的显着上调，包括p53的下游靶基因S100A8和S100A9。并且，我们在Sbds缺陷型小鼠的MPC中证实了S100a8和S100a9蛋白的过表达，这与基因剔除小鼠血浆中S100a8/9异二聚体分泌增加相关联。 进一步试验证明了S100a8/9在体外和体内环境中诱导HSPC的应激基因毒性反应是通过其经典受体，Toll样受体4（TLR4）发出信号；向基因剔除小鼠注射TLR4抗体可以部分挽救异常表型。将野生型HSPC移植到S100a9转基因小鼠中的实验证明，源自龛位的S100a8/9是诱导HSPC中应激基因毒性的充分条件。最后，为了将我们的研究结果扩展到更广泛的人类疾病，我们集中研究了最主要的白血病易感综合征，MDS。对低危（LR）-MDS患者MPC的转录组分析显示了与p53和TLR4信号通路的活化相关的S100A8和S100A9基因的显着上调。基于S100A8和S100A9蛋白在MPC中的表达（独立于现有的预后参数），这些患者可以被划分为S100龛位阳性和S100龛位阴性组。 S100龛位阳性患者表现出更恶劣的病情发展，包括白血病进展增加和无进展生存期减少。

至此，我们确定了一个全新的分子机理，即间充质龛位可以通过p53-S100A8/9-TLR4炎性信号传导诱发HSPC的应激基因毒性，并且S100A8/9的间充质表达可以预测LR-MDS患者的白血病转化。显然，除S100A8和S100A9之外，许多其他的炎性分子也在LR-MDS患者的MPC中上调； 这些发现引出了下一个重要问题，是什么诱发了间充质炎性信号?

在第三章中，我们的目标是确定在LR-MDS中诱发间充质炎症的上游调节因子。与健康供体对比，转录组分析结果显示LR-MDS患者的MPC呈现出NF-κB信号通路的特征性基因富集，以及NF-κB抑制剂α（NFKBIA）基因表达的上调，表明了间充质中NF-κB信号通路的活化是LR-MDS患者的共同特性。NFKBIA基因的表达与炎性分子的表达显着相关，其中包括NF-κB的下游靶基因。增加的 p65磷酸化（p-p65）进一步证实了LR-MDS患者间充质中NF-κB的活化。为了研究间充质NF-κB活化对HSPC的影响，我们通过在OP9基质细胞系中过表达组成性活化形式的NF-κB的激酶上游调节剂IKK2，建立了离体模型。增加的p-p65与NF-κB下游靶炎性分子基因的表达水平证实了OP9细胞系中NF-κB的活化。共培养健康供体的骨髓细胞与NF-κB活化的OP9细胞系导致了HSPC数量和功能的削减。综上，我们的实验数据证明了间充质NF-κB活化是龛位炎症的上游诱导因素，并促进了LR-MDS中的BMF症状。

在第四章中，我们重点研究了血细胞减少对BMF综合征发病和白血病进展的影响。中性粒细胞减少症，临床表现为外周血中成熟中性粒细胞数量的显著降低以及AML易感性，是SDS和其他先天性中性粒细胞减少综合征的标志。基于我们组之前建立的中性粒细胞减少症小鼠模型，我们将Cebpa启动子驱动特异性剔除Sbds的或对照组的胚胎肝脏造血细胞移植到野生型小鼠中。接受到特异性Sbds剔除细胞的小鼠罹患了严重且持续的中性粒细胞减少症，而这些小鼠HSC中的Sbds基因表达并未受到影响。与对照组相比，罹患中性粒细胞减少症小鼠的HSC数量和出现频率无显著区别，说明它们的HSC处于静息状态；并且，该结论由细胞周期分析验证。未变化的DNA损伤水平进一步表明，中性粒细胞减少没有引发HSC的DNA复制压力。与此结论一致，转录组分析显示，在中性粒细胞减少症中，HSC呈现糖酵解代谢的特征性基因富集以及降低的线粒体衍生ROS水平。与以上观察相反，在中性粒细胞减少症中，多能祖细胞（MPP）呈现出数量减少和细胞周期增加，以及活跃代谢状态的特征性基因富集。此外，中性粒细胞减少症中的HSC和MPP都呈现富集的髓细胞系定型转录程序，暗示HSC不经过分裂而直接分化为短暂增殖的MPP以补偿成熟中性粒细胞的短缺。有趣的是，通常与衰老相关的表型，比如免疫表型定义的HSC的扩增和连续再增殖能力的丧失，在罹患中性粒细胞减少症的小鼠中得到改善。潜在的分子机理可能是已知诱发干细胞衰竭的生理因子干扰素（IFN）的信号传导在中性粒细胞减少症的HSC中降低，暗示中性粒细胞可能通过诱导IFN/炎性信号传导而负向调节造血功能。另外，我们在长期暴露于中性粒细胞减少环境下的小鼠中观察到HSC龛位的重塑，具体表现为间充质基质细胞和内皮细胞的出现频率以及转录组的改变。综上，我们的数据显示HSC在慢性中性粒细胞减少症中得以保存；促进HSC保存的精确分子机理目前正在研究中。

综上所述，本论文所涵盖的研究为HSC龛位在BMF综合征发病中的作用提供了新的分子机理。我们的研究结果表明，造血细胞和骨髓微环境的基因缺陷可能协同作用从而诱导BMF和克隆性转化；炎性信号传导在该过程中是一个重要的分子机理。另外，在中性粒细胞减少症中，血细胞的短缺和对造血的需求不会导致HSC的复制压力，衰竭和恶性转化。这些发现为进一步研究理解在HSC衰竭和恶性转化的过程中不同的骨髓成分之间细胞和分子的相互作用奠定了基础。我们的科研成果预期可为LR-MDS提供新的（龛位指示的）预后方案，并可能为治疗BMF综合征和白血病提供靶向HSC龛位的新型药物靶点。

CURRICULUM VITAE

Zhen Ping was born on 4 January 1986 in Huhhot/Nei Mongol, China. After received his high school diploma from No.14 Middle School (Huhhot/Nei Mongol, China), he came to the Netherlands for higher education in 2005. He obtained his BSc diploma (double degree) in life sciences from the HAN University of Applied Sciences (Nijmegen, the Netherlands) and in applied biology from the Bonn-Rhein-Sieg University of Applied Sciences (Rheinbach, Germany) in 2009. During his BSc studies, he performed an internship under the supervision of Prof. Dr. Daniele Guardavaccaro at the Hubrecht Institute (Utrecht, the Netherlands), where he identified the ubiquitin ligase for the E2F3 transcription factor, and published his findings as the first author. Afterwards, he continued with his MSc studies at Vrije University Amsterdam (Amsterdam, the Netherlands), and earned his MSc degree in Biomolecular Sciences in 2012. During his MSc studies, he performed an internship under the supervision of Dr. Shalin Naik and Prof. Dr. Ton Schumacher at the Netherlands Cancer Institute (Amsterdam, the Netherlands), where he studied dendritic cell development by establishing a novel long-term time-lapse microscopy platform, and contributed to a cellular barcoding project in a Nature publication. Next, he performed another internship under the supervision of Dr. Qiao Zhou at Harvard University (Cambridge, the United States), where he worked on identifying the conditions to dedifferentiate adult astrocyte into neural stem cell. Through these internship experiences he discovered his main research interest in a crossover between immunology and cancer biology. In 2013, he started to pursue his PhD studies under the supervision of Prof. Dr. Marc Raaijmakers at Erasmus University Medical Center (Rotterdam, the Netherlands), where he focused on investigating the contribution of the hematopoietic stem cell niche in leukemia predisposition syndromes.

AWARDS & FELLOWSHIPS

- Top Publication Award - Dutch Hematology Congress (Arnhem, the Netherlands, 2017)

- Vrije Universiteit Fellowship Program, 2010 - 2011

LIST OF PUBLICATIONS

Zhen Ping*, Jacqueline Feyen*, Claire van Dijk, Paulina M. H. van Strien, Tamar Tak, Giulia Corradi, Remco M. Hoogenboezem, Ivo P. Touw and Marc H. G. P. Raaijmakers. *Manuscript in preparation*. ***Co-first author**

Zhen Ping*, Si Chen*, Sjoerd J. F. Hermans, Keane J. G. Kenswil, Jacqueline Feyen, Claire van Dijk, Eric M. J. Bindels, Athina M. Mylona, Niken M. Adisty, Remco M. Hoogenboezem, Mathijs A. Sanders, Eline M. P. Cremers, Dicky J. Lindenbergh-Kortleve, Janneke N. Samsom, Arjan A. van de Loosdrecht, Marc H. G. P. Raaijmakers. Activation of NF-κB driven inflammatory programs in mesenchymal elements attenuates hematopoiesis in low-risk myelodysplastic syndromes. *Leukemia*. 2019 Feb;33(2):536-541. ***Co-first author**

Keane J.G. Kenswil, Adrian C. Jaramillo, **Zhen Ping**, Si Chen, Remco M. Hoogenboezem, Maria A. Mylona, Maria N. Adisty, Eric M. J. Bindels, Pieter K. Bos, Hans Stoop, King Hong Lam, Bram van Eerden, Tom Cupedo, Marc H.G.P. Raaijmakers. Characterization of Endothelial Cells Associated with Hematopoietic Niche Formation in Humans Identifies IL-33 As an Anabolic Factor. *Cell Rep*. 2018 Jan 16;22(3):666-678.

Noemi A. Zambetti*, **Zhen Ping***, Si Chen*, Keane J.G. Kenswil, Maria A. Mylona, Mathijs A. Sanders, Remco M. Hoogenboezem, Eric M.J. Bindels, Maria N. Adisty, Paulina M.H. van Strien, Cindy S. van der Leije, Theresia M. Westers, Eline M.P. Cremers, Chiara Milanese, Pier G. Mastroberardino, Johannes P.T.M. van Leeuwen, Bram C.J. van der Eerden, Ivo P. Touw, Taco W. Kuijpers, Roland Kanaar, Arjan A. van de Loosdrecht, Thomas Vogl, Marc H.G.P. Raaijmakers. Mesenchymal Inflammation Drives Genotoxic Stress in Hematopoietic Stem Cells and Predicts Disease Evolution in Human Pre-leukemia. *Cell Stem Cell*. 2016 Nov 3;19(5):613-627. ***Co-first author**

Zhen Ping, Ratna Lim, Tarig Bashir, Michele Pagano, Daniele Guardavaccaro. APC/C (Cdh1) controls the proteasome-mediated degradation of E2F3 during cell cycle exit. *Cell Cycle*. 2012 May 15;11(10):1999-2005.

PHD PORTFOLIO

Name PhD Student: Zhen Ping
Erasmus MC Department: Hematology
Research School: Molecular Medicine (MolMed)

PhD Period: June 2013 – February 2019
Promoters: Prof.dr. H.G.P. Raaijmakers
Prof.dr. I.P. Touw

1. PhD Training	Year	ECTS
General Courses		
Photoshop and Illustrator workshop	2013	0,3
Indesign workshop	2013	0,15
Excel workshop	2014	0,3
Research management for PhD-students	2014	1
Research integrity course	2016	0,3
PhD day (4x)	2013-2016	1,2
Biomedical english writing course	2018	2
In-depth Courses and Workshops		
Biostatistics	2014	3
Molecular aspects of hematological disorders (5x)	2014-2018	3,8
Scientific Meetings Department of Hematology		
Work discussions (Weekly)	2013-2018	8
Journal club/literature discussions (bi-montly)	2013-2018	7
PhD lunch with invited speaker (Monthly)	2014-2018	2,5
Erasmus Hematology Lectures (Monthly)	2014-2018	2
National/International conferences		
Dutch Hematology Congress (3x) (Arnhem)	2014-2018	0,9
Dutch Society for Stem Cell Research Annual Meeting (2x) (Groningen, Utrecht)	2015, 2016	0,6
Molecular Medicine Day (6x) (Rotterdam)	2014-2019	1,8
European School of Haematology International Conference (Lisbon, Portugal)	2015	1
Presentations		
Departmental work discussions (Oral, 7x) (Rotterdam)	2013-2017	3,5
Journal clubs (Oral, 4x) (Rotterdam)	2014-2017	2
Molecular aspects of hematological disorders (Oral, 2x) (Rotterdam)	2016, 2018	2
Dutch Hematology Congress (2x) (Arnhem)	2017, 2018	2
2. Teaching, Supervision & Organization Activities		
Organization of PhD Day	2015	0,2
Organization and supervision PhD lunch with invited speakers	2014-2015	0,2
Total		**45,75**

WORD OF THANKS

Finally, here I am, defending my PhD dissertation after six years of a spectacular journey. This experience reminds me of a Chinese story I grew up with, *Journey to the West*. In this legendary story, a monk from the Tang dynasty was determined to obtain Buddhist sacred books from the 'Western Regions'. He left Chang'an, traveled through the vast desert in Western China, snow mountain ranges in Central Asia, and eventually arrived in India, where he studied at the ancient Buddhism university and obtained the books he was looking for. He was destined to encounter eighty-one obstacles along the way before he could achieve his goal, including the absence of established roads, falling into the traps of devils, getting stranded in temptation, having conflicts with people accompanying him, etc. Luckily, he was always assisted by amazing people (also gods and goddess) at the right moments; together with his perseverance, he ultimately got to the place where he wanted to be. Reminiscing about my PhD journey, I think many of the obstacles I have experienced and am experiencing are very similar to the story mentioned hereinabove. Fortunately, I have also met amazing people who helped me significantly to overcome those obstacles and to enjoy life at the same time. Here, I would like to express my gratitude to everyone who was part of my journey. There are so many people I would like to thank, I'm afraid it would be impossible to mention all the names properly, especially under the stress of finalizing this dissertation. Please excuse me in that case and give me an opportunity to reconnect.

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first meeting with you during the revision of our Cell Stem Cell paper when you brought the S100A9tg mice to us and took samples back to your lab in Münster for ELISA. When another member of our group was defending her PhD, you gladly came and told me to inform you in time when it's my turn. I'm very grateful that you will be here in my defense and thank you for your support in my projects regarding S100A8/9. Dear **Dr. Marije Bartels**, thank you for kindly agreeing to take part in my defense. I don't think we have met before but I will try my best to make a good first impression.

I would like to thank all the PI's in the department at present or in the past for your suggestions both in my projects and career path. Dear **Bob**, it's great to see you in office Ee-1300 from time to time, through your kind greeting and friendly smile I can feel your care and expectation for junior scientists. **Frank**, **Pieter**, **Jan**, **Eric**, **Dick**, **Moniek**, **Peter**, **Emma**, **Tom**, **Mojca**, **Ruben**, **Stefan**, your inputs in the Friday floor meetings and seminars enlightened me in shaping my scientific ideas. I enjoyed the conversations we had and learned a lot from you during the departmental events. Also, thank you for your generous support in sharing protocols, reagents, and samples over the past years!

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after completed your MD studies. Shortly after we started to work on the neutropenia project together. Your critical thinking and questioning about the project deeply impressed me. Very soon, you demonstrated your in-depth understanding of the project and you brought it further. I'm also impressed by your braveness and integrity in pointing out how things should be done not only the conventional way but also the proper way. Thanks for your practical and intellectual contribution to our project, especially during the period when I was writing. Moreover, thank you for your kind offer to proof-read my discussion chapter, finalizing figures for chapter 4, and making the Dutch summary for me! I think we made a great team together; I hope we will manage to pinpoint the molecular mechanisms of the neutropenia project and proceed to publication soon!

Keane, you are the most senior member of the group that I know next to our boss. I remember the day I came in for interview you were already there as a MSc intern student. Back then I was already impressed by your capability of firing out good scientific questions rapidly, and this got even better over the years. I appreciate that you were there, and we support each other to go through the most difficult times. For a period, there were only you and me doing the lab work in our group. I will never forget that day when I just harvested all the bone marrow samples around 8 pm but still had to run the cellular stress assays for another 5-6 hours, you came to me and offered help without me even asking. I can't remember exactly how many times you provided me with your key for the apartment in Rochussenstraat and allowed me to crash on your couch after the late-night experiments. I enjoyed sharing the small office with you, having philosophical conversations, and listening to loud music together. Your taste of music and invitation to all the concerts brought my inner party animal out. I wish you all the best in finalizing your dissertation and I look forward to being there for you in your defense soon!

Si, we spent four years together in the group, I was so happy there is someone I can speak Chinese to in the group! I have been always deeply impressed by your capability of multi-tasking. You managed multiple projects together simultaneously, you got most chapters done in the group so far, and I remember while you were writing you also did some experiments, applied for jobs and had telephone appointments in between; and eventually, you got everything done nicely! Thank you for all your help and suggestions over the past years, and your enormous contribution to the S100A8/9 project. I appreciate your kind offer to collaborate on the NF-κB project, otherwise, I might be not ready to defend my dissertation at this point. I'm grateful that our connection continues up to date; I wish you all the best with starting up in Singapore!

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always been a dedicated scientist who is on top of your subject, setting a good example for the fellow students in the group. It was not always easy to work with you under the stress for publication, but I appreciated your initiatives in smoothening it up. Memories flashback to the good times we had together, in the dinners, costume parties; and in the ESH conference in Lisbon, where we met your local friends and toured the city together! I think we did great work together in the OCS model. Thank you for your suggestions in continuing further with the Cebpa model, which turned out to be the main project in the second half of my PhD!

To the other group members in the past: **Niken**, what a small world that our paths cross again after studying together in Nijmegen! Thank you for your kind help with mouse work, FACS, and RNA-sequencing! I'm happy to see that you are enjoying life after going back to Indonesia! **Adrian**, thanks for your support when I was settling down in the group! I enjoyed all the amazing events you organized at Rochussenstraat! I hope you keep staying strong and finish your PhD work soon! **Sjoerd**, thanks for your help during the revision of the NF-κB paper! I'm impressed by your knowledge and passion for statistics! **Giulia**, thank you for your generous help in making the overexpression constructs and the co-culture work for the neutropenia project! I hope I was also helpful for the parts about Dnmt3a and Asxl1 projects in your dissertation! **Yongyi**, the day I came for interview in the group was the same day for your farewell dinner; thanks for your career suggestions! **Uttara**, you were visiting the group around the same time when I started my PhD, thanks for your help to establish the co-culture systems and nice stories about India! **Tamar**, I regret you decided to leave the group when we just got to know each other better; I enjoyed all the scientific discussions we had and the borrels we went together. Thank you for your scientific input in the neutropenia chapter, your proof-reading and comments for my discussion chapter, as well as the helpful advice regarding my defense and future career! I think you set a great example as a Postdoc in guiding junior scientists with their scientific career; I wish you all the best in your new position in LUMC!

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and life, and I hope I was patient enough to answer your questions regarding the cellular stress assays. Thank you for allowing me to crash on your couch whenever I needed it. Thank you for inviting me to your hometown in Abruzzo when I was just done with writing my dissertation, it was the best and most authentic trip I have ever had in Italy. It has been a great pleasure to meet your family and friends, having great food including Arrosticini and homemade wine! I wish you all the best in submitting your papers and graduation. **Roberto**, I'm very grateful for having the opportunity to work together with you on your Cebpa enhancer deletion mouse model in the final years of your PhD, which gave us the time to get to know each other. Many times, when I worked till late in the lab and not able to go back to Amsterdam, you offered a couch to crash. It was a surprise to find out that we share so many opinions in the philosophy of life. I admire your knowledge in the field and your capability of remembering not only the main points but also the first and last authors of each relevant literature. Your passion and vision in science enlightened me, and I enjoyed every time talking about my projects with you. Thank you for the encouragement and the faith in me, which gave me strength through the difficult times. And thank you for proofreading my introduction chapter upon short notice. I was so happy for you when you aced your defense after being through all the challenges. I sincerely hope you keep on going strong and all the best for your future career!

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"Now this is not the end. It is not even the beginning of the end.
But it is, perhaps, the end of the beginning."
—Winston Churchill