From the Department of Medicine, Huddinge
Karolinska Institutet, Stockholm, Sweden

MODELLING INEFFECTIVE ERYTHROPOIESIS IN MYELODYSPLASTIC SYNDROMES WITH RING SIDEROBLASTS

Edda María Elvarsdóttir

Karolinska Institutet

Stockholm 2019

Published by Karolinska Institutet.
Printed by Eprint AB 2019

ISBN 978-91-7831-556-7

Cover: Erythroblastic Island by Edda and Ari, 2019

Modelling Ineffective Erythropoiesis in Myelodysplastic Syndromes with Ring Sideroblasts

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

Edda María Elvarsdóttir

Principal Supervisor:

Professor Eva Hellström-Lindberg, M.D., Ph.D.
Karolinska Institutet
Department of Medicine
Center for Hematology and Regenerative Medicine

Co-supervisor(s):

Iyadh Douagi, Ph.D.
Karolinska Institutet
Department of Medicine
Center for Hematology and Regenerative Medicine

Assistant Professor Petter S. Woll, Ph.D.
Karolinska Institutet
Department of Medicine
Center for Hematology and Regenerative Medicine

Opponent:

Professor Douglas Higgs, M.D.
University of Oxford
Radcliffe Department of Medicine
The Weatherall Institute of Molecular Medicine

Examination Board:

Associate Professor Cecilia Götherström, Ph.D.
Karolinska Institutet
Department of Clinical Science, Intervention and Technology
Division of Obstetrics and Gynecology

Associate Professor Johan Flygare, M.D., Ph.D.
Lund University
Department of Laboratory Medicine
Division of Molecular Medicine and Gene Therapy

Professor Anders Österborg, M.D., Ph.D.
Karolinska Institutet
Department of Oncology-Pathology

To my parents

ABSTRACT

Myelodysplastic syndromes with ring sideroblast (MDS-RS) is a clonal hematological malignancy characterized by accumulation of iron filled erythroblasts called ring sideroblasts in the bone marrow, and recurrent somatic mutations in the splicing factor gene *SF3B1*. The disease mainly affects elderly individuals, causing severe anemia in patients for which no curative treatment exists. An important requisite to develop new treatments for MDS-RS is to identify the cell population that propagates and sustains the disease clone, as well as its functional downstream effects on erythropoiesis. Modelling the ineffective erythropoiesis of MDS-RS has been problematic both *in vitro* and *in vivo*, in particular the generation of ring sideroblasts, which has hampered functional studies of the disease. The focus of this thesis was to determine at which stage of the hematopoietic hierarchy *SF3B1* mutations originate in MDS-RS patients, and to provide experimental models recapitulating the disease phenotype, allowing for functional studies and testing of new therapeutic options.

In study **I** we demonstrated that *SF3B1* mutations in MDS-RS patients originate in the hematopoietic stem cell (HSC) compartment, before division into the myeloid and lymphoid lineages. We found clonal involvement in B-cell progenitors resulting in a negative effect on lymphoid development. Furthermore, we found that only HSCs and no other investigated progenitor populations isolated from MDS-RS patients could propagate the *SF3B1* mutated clone both *in vitro* and *in vivo*. Transplantation of HSCs from MDS-RS patients into immunodeficient mice resulted in ring sideroblast formation, providing a novel *in vivo* model to study the disease.

In study **II** we established a three-dimensional (3D) culture model capable of recapitulating healthy and aberrant terminal erythropoiesis. Suspension cultures of $CD34^+$ progenitor cells from MDS-RS patients had thus far failed to generate mature erythroid cells, including ring sideroblasts. We therefore decided to compare long term cultures of $CD34^+$ cells and mononuclear cells (MNCs) from healthy individuals and MDS-RS patients either in suspension (2D) or in 3D scaffolds that mimic the structure of the bone marrow. We found that the scaffolds provided the $CD34^+$ cells with proliferative advantage and enabled them to preserve their self-renewal potential. By comparison, the same cells did not survive beyond three weeks in 2D cultures. Additionally, the $CD34^+$ 3D cultures predominantly facilitated erythropoiesis, including enucleation and erythroid island generation. MNC cultures maintained stable proliferation for the four-week culture period, supporting multi-lineage hematopoietic differentiation and cytokine secretion relevant to erythropoiesis and MDS-RS. The $CD34^+$ 3D, MNC 3D and MNC 2D

cultures maintained the *SF3B1* mutated clone and generated ring sideroblasts *de novo* from the second week of culture, providing a novel *in vitro* model to assess therapeutic compounds aiming to alleviate the anemia in MDS-RS patients.

In study **III** we treated primary cells from healthy individuals and MDS-RS patients with luspatercept, a relatively new treatment option for MDS-RS, in the 3D model established in study **II**. Luspatercept is a transforming growth factor beta family ligand trap that has been shown to alleviate anemia in MDS-RS patients although its mechanism has not been elucidated. We found that luspatercept enhances proliferation and erythroid output of $CD34^+$ cells and MNCs from healthy individuals *in vitro*, demonstrating that it can have a direct effect on hematopoietic progenitor cells. By contrast, luspatercept had no direct effect on hemopoiesis in the MDS-RS cultures, nor did it inhibit the *SF3B1* mutated clone or ring sideroblast generation. This indicates that the drug may not directly target the disease clone although this will have to be confirmed in a larger population of responding patients. Interestingly, we found that luspatercept completely inhibited IL-6 secretion of $CD34^+$ cells from healthy individuals *in vitro*, indicating that the drug can affect cytokine secretion. Since IL-6 can have a negative effect on erythropoiesis and is upregulated in a proportion of MDS patients it is worth exploring if it is upregulated in MDS-RS patients that respond to the drug.

LIST OF SCIENTIFIC PAPERS

I. *SF3B1*-initiating Mutations in MDS-RSs Target Lymphomyeloid Hematopoietic Stem Cells
Mortera-Blanco, T., Dimitriou, M., Woll, P.S., Karimi, M., **Elvarsdottir, E**., Conte, S., Tobiasson, M., Jansson, M., Douagi, I., Moarii, M., Saft, L., Papaemmanuil, E., Jacobsen, S.E.W., Hellström-Lindberg, E. (2017) *Blood,* 130(7): 881-890

II. A Three-dimensional In Vitro Model of Erythropoiesis Recapitulates Erythroid Failure in Myelodysplastic Syndromes
Elvarsdottir, E., Mortera-Blanco, T., Dimitriou, M., Bouderlique, T., Jansson, M., Hofman, I.J.F., Conte, S., Karimi, M., Sander, B., Douagi, I., Woll, P.S., Hellström-Lindberg, E. (2019). *Leukemia,* doi: 10.1038/s41375-019-0532-7

III. *In Vitro* Effects of Luspatercept on Bone Marrow from Healthy Individuals and Patients with MDS-RS.
Elvarsdottir, E., Creignou, M., Mortera-Blanco, T., Hofman, I.J.F., Nikougoftar Zarif, M., Sander, B., Dimitriou, M., Woll, P.S., Hellström-Lindberg, E. *In manuscript*

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

3D	Three-dimensional
ABCB7	ATP Binding Cassette B7
Act-RIIB	Activin receptor type IIB
AML	Acute myeloid leukemia
ATP	Adenosine triphosphate
BFU-E	Burst-forming unit, erythroid
CFU-C	Colony-forming unit cell
CFU-E	Colony-forming unit, erythroid
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
ddPCR	Droplet digital PCR
dNTP	Deoxynucleotide triphosphate
EPO	Erythropoietin
ESC	Embryonic stem cells
ETP	Early T-cell progenitor
FACS	Fluorescence-activated cell sorting
Flt-3L	Fms like tyrosine kinase 3 ligand
G-CSF	Granulocyte colony-stimulating factor
GDF11	Growth and differentiation factor 11
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Granulocyte macrophage progenitor
HbF	Fetal hemoglobin
HSC	Hematopoietic stem cell
hiPSCs	Human induced pluripotent stem cells
HSPCs	Hematopoietic stem and progenitor cells
IL-	Interleukin-
iPSC	Induced pluripotent stem cell
LTC-CFC	Long-term culture colony forming cell
LT-HSC	Long-term HSC

MCP-1	Monocyte chemoattractant protein 1
MCP-3	Monocyte chemoattractant protein 3
MDS	Myelodysplastic syndrome
MDS-RS	MDS with ring sideroblasts
MDS-RS-MLD	MDS-RS with multilineage dysplasia
MDS-RS-SLD	MDS-RS with single lineage dysplasia
MEP	Megakaryocyte erythroid progenitor
MNC	Mononuclear cell
MPP	Multipotent progenitor
PB	Peripheral blood
PCR	Polymerase chain reaction
PPi	Pyrophosphate
proB	B-cell progenitor
RBC	Red blood cell
SCF	Stem cell factor
SF3B1	Splicing factor 3B subunit 1
ST-HSC	Short-term HSC
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TNFα	Tumor necrosis factor alpha
UCB	Umbilical cord blood
VAF	Variant allele frequency
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 HEMATOPOIESIS

Hematopoiesis is classically defined as a stepwise process of the formation of all morphologically and functionally distinct blood cells originating from hematopoietic stem cells (HSCs) and essentially takes place within the bone marrow of adult human's. HSCs are pluripotent stem cells that can be divided into two sub-groups, long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs) [1-3]. The LT-HSCs are a rare, mostly dormant population that can continuously self-renew, while ST-HSCs can self-renew for a short time (8-12 weeks) before they differentiate into multipotent progenitors (MPPs), a population with a reduced self-renewal potential [4]. Further down the hierarchy the MPPs differentiate into common lymphoid progenitors (CLPs), which give rise to T lymphocytes, B lymphocytes and natural killer-cells [5], or common myeloid progenitors (CMPs), which give rise to the myeloid lineage, including the megakaryocyte erythroid progenitors (MEPs) and the granulocyte macrophage progenitors (GMPs) (Figure 1) [6].

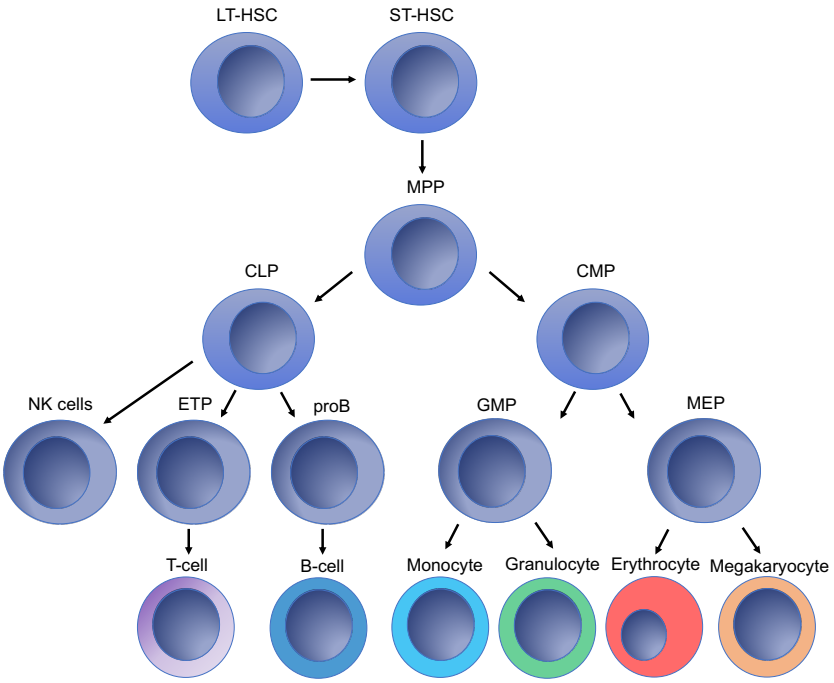


Figure 1 The classical hierarchy of adult human hematopoiesis. Long term hematopoietic stem cell (LT-HSC), short term hematopoietic stem cell (ST-HSC), multipotent progenitor (MPP), common lymphoid progenitor (CLP), common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP), megakaryocyte/erythroid progenitor (MEP), natural killer cells (NK cells), early T-cell progenitor (ETP), B-cell progenitor (proB). The figure is adapted from Meyer 2017 [7].

The notion of hematopoiesis as a set, stepwise process has continuously been debated. As an example, megakaryocytes have been reported to directly differentiate from HSCs, indicating that they can bypass the stages of MPPs, CMPs and MEPs [8, 9]. Additionally, it has been suggested that hematopoiesis is a continuous rather than step-wise process [10-12] and that the physiological conditions during experimentation, such as stress as a result of *in vitro* culture or hematopoietic reconstitution during transplantation, might differ from steady state hematopoiesis (as reviewed by Crisan and Dzierzak, 2017) [13].

1.2 ERYTHROPOIESIS

Erythropoiesis is the differentiation and maturation of erythroid cells originating from the HSCs and takes place successively in the yolk sac, the fetal liver and the bone marrow during mammalian development. The first committed erythroid progenitor cells derive from MEPs and are distinguished by their capacity to generate colonies *in vitro*. The earliest erythroid precursor is the BFU-E (burst-forming unit, erythroid), which generates multi-clustered colonies in semisolid medium, followed by the CFU-E (colony-forming unit, erythroid), which can generate a smaller cluster of erythroid cells that mature into erythrocytes [14, 15]. Additionally, BFU-Es and CFU-Es can be distinguished based on growth factor requirements where the former depend on stem cell factor (SCF) and interleukin-3 (IL-3) signaling and the latter depend on erythropoietin (EPO), the major factor regulating erythropoiesis. The first morphologically distinct erythroid cells are pro-erythroblasts that derive from the CFU-Es and are succeeded by basophilic erythroblasts, polychromatic erythroblasts and orthochromatic erythroblasts that enucleate to form reticulocytes, which are released into the blood stream where they finally mature into erythrocytes (Figure 2).

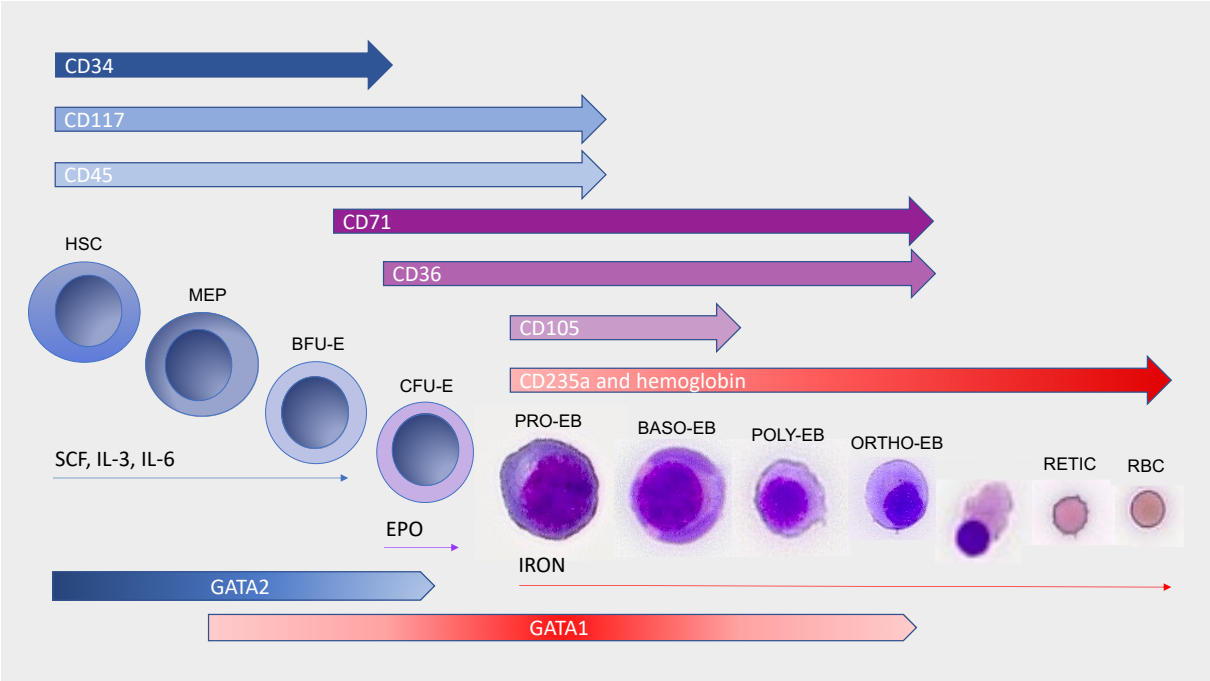


Figure 2 Erythroid maturation. Arrows represent cell surface marker/receptor expression and hemoglobin production. GATA1 and GATA2 are transcription factors and SCF, IL-3, IL-6 and EPO are required growth factors. Proerythroblasts (PRO-EB), basophilic erythroblasts (BASO-EB), polychromatic erythroblasts (POLY-EB), orthochromatic erythroblasts (ORTHO-EB), reticulocytes (RETIC) and enucleated erythrocytes/red blood cells (RBC).

These cells are less reliant on EPO than the BFU-Es and CFU-Es, demonstrated by the loss of the EPO receptor, and strongly iron dependent [16]. Additionally, erythropoiesis is heavily influenced by secreted factors such as insulin, insulin-like growth factor, activin, interleukin 10 (IL-10) and angiotensin II which have been reported to have a positive effect on erythropoiesis [17-20]. On the other hand transforming growth factor β (TGFβ), growth and differentiation factor 11 (GDF11), interferon-γ, tumor necrosis factor α (TNFα) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) have a negative effect on erythropoiesis [21, 22]. Transcription factors also play an important role, where GATA2 is highly expressed in hematopoietic stem and progenitor cells and regulates their proliferation and maintenance [23, 24], while GATA1 drives terminal erythroid differentiation [25-27]. During this maturation process the cells go through morphological changes where they gradually decrease in size, go through chromatin condensation, hemoglobin synthesis, enucleation, lose organelles and gain the biconcave disk shape [14, 15, 28].

Dividing the different maturation stages via cell surface marker or receptor expression is a common practice when studying erythropoiesis. BFU-Es have been found to express the

glycoprotein CD34, the protein tyrosine phosphatase CD45 and the transferrin receptor (CD71) while CFU-Es are negative for CD34 and rather express the glycoprotein CD36 and CD71 to a higher extent [29]. Pro-erythroblasts can be distinguished by endoglin (CD105), CD45 and c-kit (CD117) expression while being negative for CD34. Basophilic, polychromatic and orthochromatic erythroblasts are negative for CD45 but express CD36, CD71 and glycophorin a (CD235a) [30, 31]. Distinguishing between the different erythroblasts with cell surface markers has not reached consensus but using Ter119 (mouse equivalent to CD235a) coupled with the glycoprotein CD44 and forward scatter (FSC) intensity has been a successful method in mice [32, 33]. In humans adding CD36 and CD235a to the CD44/FSC expression analysis allows for a similar distinction [34]. Enucleated erythrocytes can be distinguished using a membrane based viability dye coupled with CD235a positivity and lack of nuclear staining [35], but distinguishing between reticulocytes and enucleated red blood cells (RBCs) remains problematic. The strategies for distinguishing between erythroid cells are evolving, with varying combination of cell surface markers being reported, and hopefully future studies of overlapping markers will enable a purer isolation of each and every step.

1.3 THE BONE MARROW MICROENVIRONMENT

The bone marrow is a spongy tissue made out of interlocked pores of different sizes, facilitating the formation of diverse cellular niches. It is a complex hematopoietic inductive microenvironment that relies on the interplay of many different cell types as well as acellular components. In addition to cells of the hematopoietic lineage the bone marrow includes cells of the mesenchymal stem cell lineage, such as the bone forming osteoblasts, the bone resorbing osteoclast, the cartilage forming chondrocytes, the lipid-storing adipocytes and the collagen fiber producing fibroblast. Other cells found in the bone marrow microenvironment are vascular endothelial cells, CAR (CXCL12 abundant reticular cells, a chemokine required for HSC maintenance and retention in the bone marrow [36]) cells and sympathetic neurons. These cells play an important role in providing ligands and cytokines for inducing proliferation, survival and differentiation. A crucial cellular component for erythropoiesis found in the bone marrow microenvironment is the erythroblastic island, where 5-30 maturing erythroblasts surround a central macrophage (Figure 3) [37, 38].

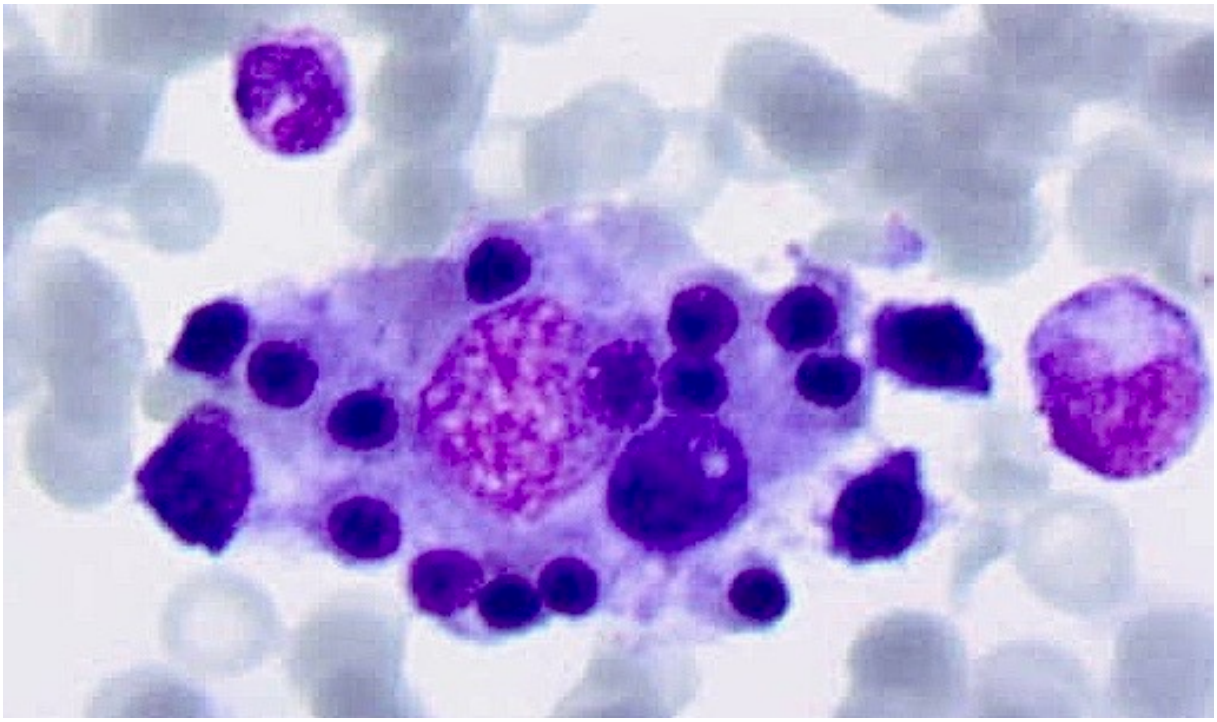


Figure 3 An erythroblastic island from a bone marrow biopsy composed of a macrophage surrounded by maturing erythroid cells. Figure generously provided by Birgitta Sander.

These islands are thought to play an important role in terminal erythroid differentiation where extruded nuclei from developing erythroblasts are phagocytosed by the macrophage [37]. It has been shown that they enhance erythroid proliferation and suggested that the macrophage functions as a nurse cell that provides nutrients and iron for heme synthesis to the erythroblasts [39-41]. Although macrophage ablation experiments have shown that steady state erythropoiesis is not completely reliant on erythroblastic islands, a quick response to increase erythroid output, for an example in the case of anemia, seem to depend on their presence [42]. In addition to the cellular components a variety of acellular factors contribute to the bone marrow microenvironment. These include growth factors and cytokines, extracellular matrix proteins like collagen and fibronectin, minerals like calcium, blood vessels and physical factors like shear stress, oxygen tension and temperature (as reviewed by Panoskaltsis *et al,* 2005 and Wang *et al,* 2011) [43, 44]. On top of that the structure of the bone marrow, and the position of different cell types within the pores of the bone marrow, contribute to concentration gradients of secreted factors that can have an effect on hematopoietic proliferation and differentiation [45, 46].

Failure of the bone marrow microenvironment and its niches to maintain functional hematopoiesis is thought to contribute to pathological conditions. It has been shown that

manipulation of osteoblasts in mice can induce myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) like phenotypes and that patient derived stromal cells can promote malignant behavior of human MDS cells [47, 48]. Ageing also has an effect, where age related inflammation of the bone marrow microenvironment can induce ineffective erythropoiesis [49].

1.4 *IN VITRO* MODELS OF ERYTHROPOIESIS

Oxygen carrying RBCs are the most abundant cell type in the body. In adults, more than two million erythrocytes are released from the bone marrow into the bloodstream every second [50]. Despite this robust generation *in vivo* the *in vitro* modelling of terminal erythropoiesis has remained a challenge in regards of limited proliferation, difficulties in producing red cells that express adult hemoglobin and low amounts of enucleated erythrocytes [51]. To overcome these hurdles, both intrinsic and extrinsic factors regulating erythropoiesis have to be coordinated. The *in vitro* models of erythropoiesis that are most commonly used today can be divided into cultures using hematopoietic stem and progenitor cells (HSPCs), embryonic stem cells (ESCs) or human induced pluripotent stem cells (hiPSCs), where immortalized cell lines and three-dimensional (3D) cultures represent exciting options for future studies (Table 1).

Table 1: Overview of erythroid culture models

Cell type	Source	*Feeder cells	Hemoglobin	ER	Reference
HSPCs	BM, PB, UCB	Yes	Fetal and/or adult	>90%	Huang *et al,* 2018
ESCs	ESC lines	Yes	Fetal and adult	>60%	Lu *et al,* 2013
hiPSCs	Somatic cells	Yes	Fetal and/or adult	26%	Kobari *et al,* 2012
Immortalized cell lines	hiPSCs, UCB, HSPCs, ESCs	No	Fetal and/or adult	30%	Trakarnsanga *et al,* 2017
3D culture	hiPSCs, UCB, HSPCs, ESCs	No	Fetal and/or adult	>90%	Lee *et al,* 2015

*Feeder layer or stromal cells traditionally used. ER= maximum reported enucleation rate, references are given for ER. Adapted from Sun *et al,* 2018.

1.4.1 *In vitro* erythropoiesis from HSPCs

HSPCs used in erythroid cultures usually originate from the bone marrow, peripheral blood (PB) or umbilical cord blood (UCB). Using UCB in cultures facilitates higher production of progenitor cells than using HSPCs derived from PB or bone marrow. However, the cells produced from

UCB mostly contain fetal hemoglobin (HbF) [52, 53]. Generating mature erythrocytes from HSPCs in suspension cultures has been challenging [53, 54] but possible if co-cultured with stromal cells [52, 55, 56] or when erythroblastic islands are included in the culture [57]. Co-culture systems have enabled high production of mature erythrocytes but difficulties in purity of isolation, presence of foreign human or animal antigens and variable efficiency of progenitor expansion persist [58]. In 2011, Giarratana and associates developed a feeder-free culture system enabling production of up to 80% reticulocytes from PB HSPCs capable of maturing to red blood cells after injection into immunocompromised mice [59]. Following this, enucleated erythrocytes have been produced from HSPCs with >90% enucleation ratio via multiphase protocols with complex, optimized growth factor and cytokine combinations, either with stromal co-culture or including serum [60-62]. Modelling erythropoiesis by culturing primary HSPCs from patient derived PB or bone marrow, preferably in a more simplified medium and without the need for stroma, would be ideal to study diseases with dysregulated erythropoiesis

1.4.2 *In vitro* erythropoiesis from ESCs

Human ESCs are pluripotent stem cells generated after *in vitro* fertilization. While these cells can be propagated and expanded indefinitely *in vitro,* ethical issues regarding their origin may arise. Expansion and differentiation of ESCs towards erythropoiesis have mostly relied on non-human stromal or feeder cell co-cultures, and have resulted in erythrocytes with HbF (as reviewed by Christaki *et al,* 2019) [58]. In 2008, protocols for differentiating human ESCs into enucleated erythroid cells capable of adult hemoglobin expression were reported, yet relying on stromal co-culture [55, 63]. In 2013 a feeder free system for producing hematopoietic cells from human ESCs via 3D microcarriers, with potential for further differentiation, was described [64]. While useful for studying healthy erythropoiesis, genetic manipulation would be required to mimic diseases, making ESCs not the ideal source to study dysregulated erythropoiesis.

1.4.3 *In vitro* erythropoiesis from hiPSCs

In 2006, Yamanaka and associates described the generation of iPSCs by reprogramming somatic cells from mice into pluripotent stem cells via forced expression of transcription factors Oct3/4, Sox2, c-Myc and Klf4 [65]. Since iPSCs can be produced from any cell type, including primary cells from patients, these cultures provide a great opportunity to study dysregulated

erythropoiesis. Similar to ESCs they are an unlimited cellular resource and their expansion mostly relies on stromal or feeder layer co-culture (as reviewed by Christaki *et al,* 2019) [58]. Lapillonne and associates produced mature erythrocytes using hiPSCs derived from human fibroblasts in 2010, albeit with low enucleation rates (around 10%) and only expressing HbF [66]. In 2012, nucleated erythroblasts generated from hiPSCs went through terminal erythropoiesis with adult hemoglobin expression when injected into immunodeficient mice [67]. More recently, mature erythrocytes expressing adult hemoglobin have been generated from fibroblast-, bone marrow stromal cell- and PB erythroid progenitor-derived hiPSCs [68, 69]. This exciting new alternative still suffers from low enucleation rates that hopefully will be overcome in the close future.

1.4.4 Immortalized erythroid cell lines

Another exciting approach is to generate immortalized erythroid cell lines capable of terminal erythroid maturation. Production of functional enucleated red blood cells from immortalized human erythroid progenitor cell lines derived from hiPSCs and UCB was reported for the first time in 2013 [70]. The cell lines mostly facilitated production of erythroid cells with fetal globin expression with a low efficiency in enucleation. Following this, ESCs were used to produce immortalized erythroid progenitor cell lines, although these cells only expressed fetal hemoglobin and could only enucleate after injection into immunodeficient mice [71]. Since then immortalized cell lines produced from bone marrow CD34 positive cells, capable of producing reticulocytes with adult hemoglobin have been produced [72]. Recently, bioreactor expansion of immortalized CD71 and CD235a positive erythroblast from adult PB have been reported [73]. These type of cell lines could be a valuable source for *ex vivo* generation of red cells for transfusion purposes in the future.

1.4.5 *Ex vivo* bone marrow mimicry

Interestingly, none of the above-mentioned *ex vivo* models take into account the three-dimensional structure and interactions of the human bone marrow. The fact that transfusions of cultured cells into mice seem to facilitate higher amounts of RBC formation highlights the importance of mimicking the *in vivo* microenvironment when modelling erythropoiesis. Three-dimensional culture systems consisting of highly porous scaffolds can facilitate hematopoietic niche formations by mimicking the biological and mechanical function of the extracellular matrix [74, 75]. These scaffolds can be made of natural materials, such as microporous collagen

microcarriers shown to facilitate terminal erythropoiesis of CB [76], or synthetic polymers like PMVE-alt-MA that can maintain pluripotency of HSPCs *in vitro* [77]. Additionally, biodegradable porous scaffolds such as those made out of polyurethane have been shown to sustain cytokine-free expansion of UCB *in vitro* [78]. Although not well established for erythroid culture specifically these types of 3D cultures are an exciting option for mimicking the physiological factors present in erythropoiesis *in vivo*.

1.5 MYELODYSPLASTC SYNDROMES

Myelodysplastic syndromes (MDS) constitute a heterogeneous group of clonal myeloid neoplasms originating in the HSCs, characterized by ineffective hematopoiesis, morphological dysplasia, cytopenia, and bone marrow failure [79]. MDS is associated with risk for developing acute AML and survival after diagnosis varies from a few months up to ten years [79, 80]. MDS is mainly a disease of the elderly with an increase of incidence after 60 years of age [80]. The most common feature of MDS is anemia caused by ineffective erythropoiesis that can range from mild to severe [81]. The World Health Organization (WHO) classifies MDS according to degree of dysplasia and blast percentage along with morphology, immune-phenotype and genetic, molecular and clinical features, into seven different subgroups. These are MDS with single lineage dysplasia (MDS-SLD), MDS with multilineage dysplasia (MDS-MLD), MDS with ring sideroblasts (MDS-RS), which can be further divided into MDS-RS with single or multilineage dysplasia (MDS-RS-SLD or MDS-RS-MLD respectively), MDS with isolated del(5q), MDS with excess blasts (MDS-EB), MDS unclassifiable (MDS-U) and refractory cytopenia of childhood (Table 2) [82].

Table 2: The 2016 revision to the WHO classification of MDS

Disease	Dysplastic lineages	Cytopenias*	RS as % of marrow erythroid elements	BM and PB blasts	Cytogenetics by conventional karyotype analysis
MDS-SLD	1	1 or 2	<15%/<5%**	BM <5%, PB <1%, no Auer rods	Any, unless fulfills criteria for MDS with isolated del(5q)
MDS-MLD	2 or 3	1-3	<15%/<5%**	BM <5%, PB <1%, no Auer rods	Any, unless fulfills criteria for MDS with isolated del(5q)
MDS-RS					
MDS-RS-SLD	1	1 or 2	≥15%/≥5%**	BM <5%, PB <1%, no Auer rods	Any, unless fulfills criteria for MDS with isolated del(5q)
MDS-RS-MLD	2 or 3	1-3	≥15%/≥5%**	BM <5%, PB <1%, no Auer rods	Any, unless fulfills criteria for MDS with isolated del(5q)
MDS - del(5q)	1-3	1-2	None or any	BM <5%, PB <1%, no Auer rods	del(5q) alone or with 1 additional abnormality except -7 or del (7q)
MDS-EB					
MDS-EB-1	0-3	1-3	None or any	BM 5-9% or PBv2-4%, no Auer rods	Any
MDS-EB-2	0-3	1-3	None or any	BM 10-19% or PB 5-9% or Auer rod	Any
MDS-U					
with 1% blood blasts	1-3	1-3	None or any	BM <5%, PB = 1%‡, no Auer rods	Any
with single lineage dysplasia and pancytopenia	1	3	None or any	BM <5%, PB <1%, no Auer rods	Any
based on defining cytogenetic abnormality	0	1-3	<15%§	BM <5%, <1%, no Auer rods	MDS-defining abnormality
Refractory cytopenia of childhood	1-3	1-3	None	BM <5%, PB <2%	Any

(*) Cytopenias as defined as: hemoglobin,10 g/dl; platelet count, <100 x 10^9/L; and absolute neutrophil count, <1.8 x 10^9/L. Rarely, MDS may present with mild anemia or thrombocytopenia above these levels. PB monocytes must be <1 x 10^9/L; (**) If *SF3B1* mutation is present. (‡) One percent PB blasts must be recorded on at least 2 separate occasions. (§) Cases with $15% ring sideroblasts by definition have significant erythroid dysplasia, and are classified as MDS-RS-SLD. Adapted from Arber *et al,* 2016 [82].

These syndromes can be further grouped according to the revised International Prognostic Scoring System, which is used to predict risk of leukemic transformation and survival. This system is based on percentage of marrow blasts, bone marrow cytogenetics and number and degree of cytopenia where MDS can be classified into five different risk groups; very low, low, intermediate, high and very high [83].

1.5.1 Mutational landscape in MDS

Several large cohorts of MDS patients have been sequenced with next generation sequencing approaches. These studies have shown that 80-90% of patients with MDS have oncogenic mutations and that leukemia free survival has an inversed correlation with the complexity of the sub-clone [84-86]. These recurrent mutations have a prognostic value and mutational screening will likely be incorporated into future WHO classification of MDS [87]. Genes that are recurrently mutated in MDS are implicated in signal transduction, DNA modification, chromatin regulation and RNA splicing and the most commonly mutated genes are *SF3B1*, *TET2*, *SRSF2, ASXL1, DNMT3A, RUNX1, U2AF1, TP53, EZH2* and *IDH2* [85-87]. In 2011 it was established that mutations of the RNA splicing machinery, like in *U2AF1*, *ZRSR2*, *SRSF2* and *SF3B1*, are frequent and specific to myeloid neoplasms [88, 89]. These mutations most likely occur early in MDS development and might therefore be important in determining the evolution of the disease [85, 90]. *SF3B1* (splicing factor 3B subunit 1) is most commonly mutated in MDS-RS while mutations of epigenetic regulators are more prevalent in other subtypes of MDS [87].

1.6 MYELODYSPLASTC SYNDROMES WITH RING SIDEROBLASTS

The MDS subgroup myelodysplastic syndrome with ring sideroblasts or MDS-RS was first described by Bjorkman in 1956 [91] and was consequently categorized as idiopathic acquired sideroblastic anemia by the French American British classification [92]. It was redefined by the International Agency for Research on cancer in 2011 as refractory anemia with ring sideroblasts (RARS) or refractory cytopenia with multi-lineage dysplasia and ring sideroblasts (RCMD-RS) [93]. The 2016 revision of the WHO classification of MDS took into account that the percentage of ring sideroblasts is not relevant in regards of prognosis. Therefore, patients

are now diagnosed with MDS-RS if they have *SF3B1* mutations and ring sideroblasts, comprising 5% of nucleated erythroid cells in the bone marrow, or at least 15% if no *SF3B1* mutation is detected. MDS-RS is further grouped into MDS-RS-SLD (previously RARS) and MDS-RS-MLD (previously RCMD) [82]. MDS-RS is characterized by ring sideroblasts in the bone marrow, causing severe anemia and erythroid dysplasia in patients, while they have a relatively good survival chance and are at a low risk of progression to AML. Ring sideroblasts are erythroblasts in which iron accumulates in the mitochondria in the form of mitochondrial ferritin, where at least 5 iron granules are perinuclear and either surround the nucleus or encompass one third of the area surrounding it (Figure 3) [79, 94].

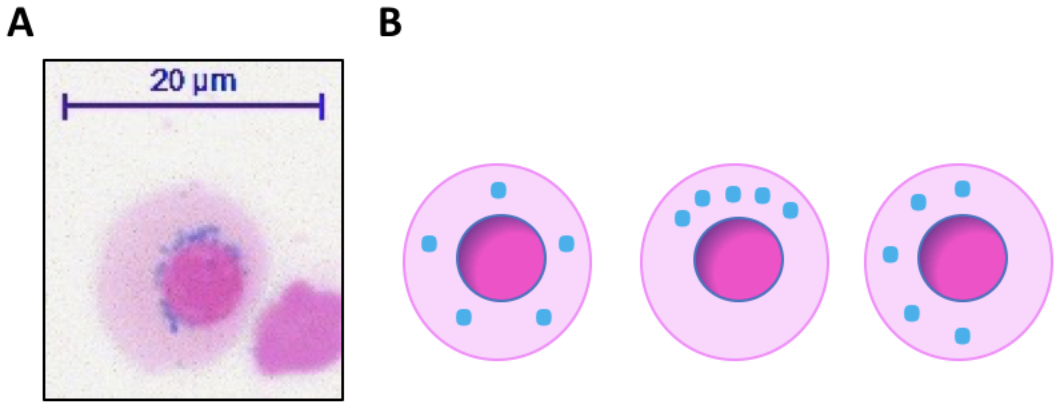


Figure 4 Ring sideroblasts. A) A ring sideroblast fixed and stained with Perl´s Prussian blue (scale bar; 20 µm). B) Examples of iron granule distribution in ring sideroblasts where the blue circles represent the iron. The figure is adapted from Mufti *et al*. 2008 [79].

In earlier studies, we have shown that MDS-RS erythroblasts release cytochrome *c* from the mitochondrial intermembrane space, which results in activation of caspase-9 and apoptosis [95]. The resulting erythroid dysplasia is in the form of hyperplastic erythropoiesis, where there is an increased percentage of erythroblasts in the bone marrow, followed by apoptosis just before terminal maturation into reticulocytes [96]. Here, growth factors such as EPO and granulocyte colony-stimulating factor (G-CSF), can both act via inhibition of apoptosis of myelodysplastic progenitors or via selection of cytogenetically normal progenitors [95, 97].

1.6.1 *SF3B1* mutations in MDS-RS

The most frequent mutations in MDS-RS patients are in splicing factors (74.5%), DNA

methylators (33%), chromatin modifiers (14.4%) and in transcription factor RUNX1 (11.5%) [98]. Up to 90% of MDS-RS patients have somatic mutations in the splicing factor gene *SF3B1,* which gives a 97.7% positive predictive value for ring sideroblast formation in the bone marrow [98, 99]. *SF3B1* wild type patients have a high prevalence of TP53 mutations leading to worse outcome [98]. *SF3B1* is located at chromosome 2q33 and encodes for a core component of the RNA splicing machinery [88]. The most common recurrent mutations of *SF3B1* in MDS-RS patients affect amino acids K700 (45-68%), H662 (10%), E622 (7%) and R625 (6%) and they lead to altered selection of 3´splice sites [85, 88, 99, 100]. Mutations of splice factors like *SF3B1* can lead to mis-splicing of RNA transcripts and thereby altered gene expression. For an example there is a difference between MDS-RS patients and healthy individuals in the exon usage of *ABCB7*, the gene encoding for an iron transporter downregulated in MDS-RS patients [94, 101, 102]. Additionally, silencing of *SF3B1* in K562 cells results in ABCB7 down regulation [102]. *SF3B1* mutated MDS-RS progenitor cells also have impaired splicing of genes involved in hemoglobin synthesis and are associated with down regulation of mitochondrial genes [88, 96]. Recently it has been shown that the erythroid hormone erythroferrone has an alternative transcript in patients with MDS-RS. This leads to generation of a variant protein maintaining suppression of hepcidin transcription, likely being the cause of increased iron loading in patients [103]. Furthermore, *SF3B1* mutated erythroid progenitors from zebrafish display G0/G1 cell-cycle arrest with a normal expression of regulators of erythropoiesis, such as gata1, globin genes and heme biosynthetic factors, while upregulating genes of the TGFβ pathway. Inhibition of TGFβ signaling released the cell cycle block, leading to enhanced anemia, indicating that the TGFβ induced cell-cycle arrest could be protective for *SF3B1* mutated erythroid cells [104].

The average variant allele frequency (VAF) of *SF3B1* mutations in MDS-RS patients is around 40% and these mutations provide a competitive clonal advantage over the wild type clone [98]. *SF3B1* mutations can be found in healthy elderly individuals representing a selection pressure for premalignant clonal expansion driven by the aging hematopoietic system [105]. *SF3B1* has been reported to mutate early in disease progression although it can occur as a secondary event [85, 106]. Elucidating at what stage of the hematopoietic hierarchy *SF3B1* mutations originate, identifying the cell capable of propagating and sustaining the *SF3B1* mutated clone and the functional down-stream effects on erythropoiesis will be key for understanding aberrant erythropoiesis and developing new treatments for MDS-RS (Figure 5).

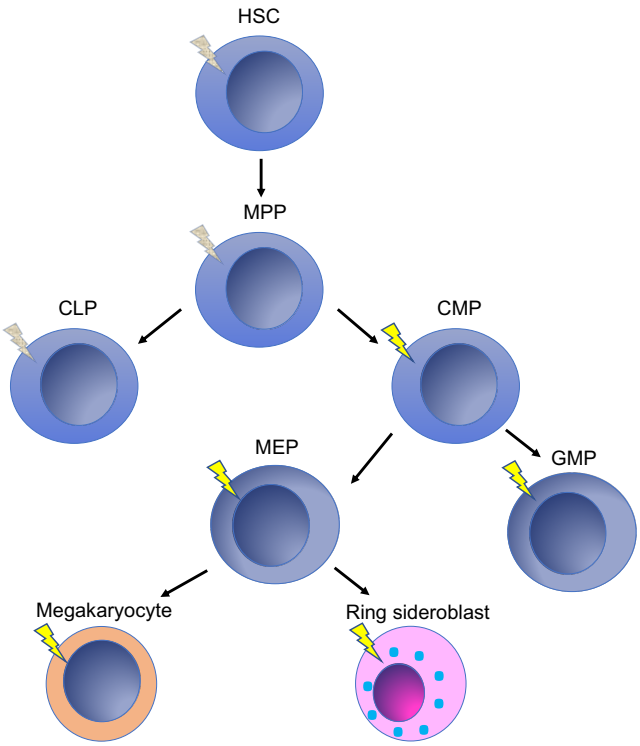


Figure 5 Hematopoiesis in MDS-RS. Yellow lightning represents *SF3B1* mutation, gray lightning represents possible clonal involvement. Hematopoietic stem cell (HSC), multipotent progenitor (MPP), common lymphoid progenitor (CLP), common myeloid progenitor (CMP), granulocyte/macrophage progenitor (GMP) and megakaryocyte/erythroid progenitor (MEP). The figure is adapted from Meyer 2017 [7].

1.6.2 Experimental models of MDS-RS

The most commonly used *in vitro* models for studying MDS-RS are suspension cultures of $CD34^+$ progenitor cells that allow for differentiation to erythroid precursors, but do not consistently generate ring sideroblasts or mature erythroid cells [94, 95, 97, 107]. While initial mouse studies indicated that *SF3B1* haploinsufficiency resulted in increased ring sideroblast formation in the bone marrow of mice [108, 109], subsequent *SF3B1*^{K700E} conditional knock-in mice did not support this observation [110, 111]. In fact, the murine orthologues of genes associated with ring sideroblasts in humans, such as *ABCB7,* were not mis-spliced, indicating a poor conservation of splice sites between the species [110, 111]. With that in mind new experimental models that mimic the dysregulated erythropoiesis of MDS-RS, including generation of ring sideroblasts, are needed.

1.6.3 MDS-RS treatment

MDS-RS patients that present with moderate to severe anemia are usually treated with recombinant human EPO and in some cases in combination with G-CSF. This treatment normalizes or improves the anemia and reduces the number of apoptotic bone marrow precursors by blocking cytochrome c release during erythroid differentiation [95, 97]. However, some patients never respond and almost 50% of patients relapse after a median of two to three years. Such patients usually become transfusion dependent which is associated with a worsening of survival and quality of life [112-115]. Additionally, chronic transfusion therapy can lead to iron overload which is damaging to organs such as the heart and liver [116] and results in a need for therapy with iron chelation agents like deferoxamine and deferasirox [116]. The mechanism for EPO resistance is unknown but does not seem to be related to disease progression to higher-risk MDS or leukemia [114]. EPO enhances the oxygen carrying capacity of blood cells through stimulation of the EPO receptor [117], which is important for survival, proliferation and differentiation of erythroid progenitors, but not for late stage erythropoiesis where the anemia in MDS-RS patients originates [28, 118]. We are therefore in need of alternative treatment options targeting mature erythroblasts.

1.6.4 TGFβ superfamily ligand traps; new treatment option for MDS-RS patients

In 2014, two studies were published describing TGFβ superfamily ligand traps as a new alternative to alleviate anemia [21, 22]. Ligand traps are molecules that bind and block the interaction of ligands to their receptors and therefore inhibit their signaling [119]. These drugs were first investigated in order to improve bone mineral density in menopausal women but surprisingly resulted in increased levels of RBCs and hemoglobin [119, 120]. An activin receptor type IIA ligand trap named sotatercept was shown to alleviate anemia in a β-Thalassemia mouse model [21], while luspatercept, an activin receptor type IIB (Act-RIIB) ligand trap, was shown to alleviate anemia in a high risk MDS mouse model [22]. Luspatercept is a fusion protein comprised of the extracellular domain of the human Act-RIIB, modified to reduce activin binding, with the Fc domain of the human immunoglobulin G1 antibody (Figure 5) [22, 119].

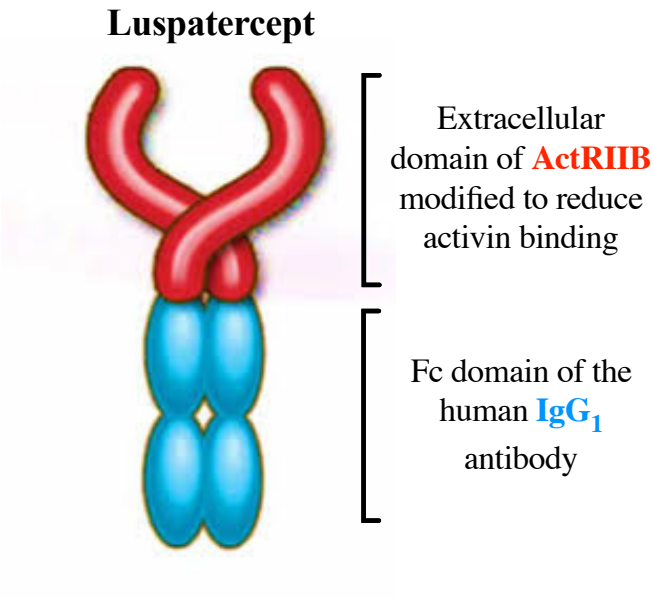


Figure 6 The TGFβ superfamily ligand trap luspatercept. Activin receptor type IIB (ActRIIB) and human immunoglobulin G1 (IgG_1). The figure is adapted from Fenaux *et al,* 2019 [121].

Treatment with a combination of luspatercept and EPO in wild type mice has been reported to give enhanced maturation of basophilic erythroblasts compared to administering the drugs separately [22]. A phase II trial of MDS patients treated with luspatercept demonstrated that the drug is well tolerated and effective in lower-risk MDS, with a greater response rate in MDS-RS patients [122]. Preliminary results reported at the 2018 ASH meeting from an ongoing phase III trial demonstrated significantly decreased transfusion burden in MDS-RS patients with 37.9% of patients becoming transfusion independent [123].

Both sotatercept and luspatercept have been shown to bind to GDF11 [21, 22], a cytokine reported to be elevated in the serum of MDS patients [22, 124]. GDF11 is thought to be a key player in erythropoiesis where it inhibits differentiation but maintains survival of erythroid progenitors [22]. Previously, it was speculated that GDF11 expressing erythroid progenitors accumulated and maintained their own survival in MDS-RS patients, and that luspatercept binding GDF11 would allow the erythroid progenitors to mature and thereby alleviate anemia in patients [22]. Recently this hypothesis has been questioned since the positive effect of luspatercept on erythropoiesis can also be found in healthy humans and mice, where GDF11 overexpression has not been reported [22, 119]. Indeed in 2019 Guerra and associates demonstrated that a pan cellular deletion of GDF11 in a β-Thalassemia mouse model did not have any effects on erythropoiesis and that treating said mice with RAP-536 (the mouse analog

of luspatercept) resulted in a significant increase in RBCs, hemoglobin and hematocrit, showing that luspatercept does not primarily work through binding of GDF11 [125]. *In vitro* studies of luspatercept/RAP-536 have mostly consisted of suspension culture of erythroid progenitor cells from healthy individuals and have not succeeded in recapitulating the effects luspatercept has on erythropoiesis [22, 125]. This might be explained by the fact that these cultures are suboptimal at reproducing terminal erythropoiesis. Luspatercept may also have a secondary effect on erythropoiesis by binding cytokines secreted by the bone marrow stroma. This seems to be the case for sotatercept, where media extracted from stromal cells treated with the drug could restore the disrupted erythropoiesis of conditioned $CD34^+$ cells *in vitro* [126]. Additionally, the effect luspatercept has on the *SF3B1* mutated disease clone and ring sideroblast generation have not been reported. Therefore, investigations into the functional effect of luspatercept on MDS-RS erythropoiesis are needed.

2 AIM OF THE THESIS

The main research topic of this thesis is to study the dysregulated erythropoiesis of MDS-RS where the aim was to:

- Characterize at what stage of the MDS-RS hematopoietic hierarchy *SF3B1* mutations originate and identify the cellular compartment capable of propagating and sustaining the mutated clone

- Establish novel experimental models that mimic healthy and MDS-RS erythropoiesis, including generation of ring sideroblasts, to enable functional studies of the downstream effects of *SF3B1* mutations

- Study the effects of luspatercept on MDS-RS erythropoiesis by exploring if the drug alters the size of the *SF3B1* mutated clone or percentage of ring sideroblasts, and if it has a direct effect on erythroid cells or secondary via binding ligands secreted by the bone marrow stroma

3 METHODOLOGICAL APPROACHES

The experimental techniques used to produce data for this thesis are described in detail in study **I-III**. Therefore, only the principal methods and considerations are described below.

3.1 CELL ISOLATION AND CULTURE CONDITIONS

Bone marrow aspirates from healthy individuals and MDS-RS patients were collected at Karolinska University Hospital, Sweden, after obtaining informed consent from the study participants and with approval by the Ethics Research Committee at Karolinska Institutet (2010/427-31/1 and 2011/1257-31/1). Mononuclear cells (MNCs) were separated via density gradient centrifugation (Lymphoprep^{TM}) and a proportion of the MNCs were further purified into $CD34^+$ progenitor cells via magnetic-activated cell sorting. To maximize the quantity of isolated cells, $CD34^+$ purification was performed using the single separation protocol, resulting in purity of around 80%. The isolated MNCs and $CD34^+$ cells were then either seeded into suspension cultures (2D) or into polyurethane scaffolds (3D culture). The MNCs were seeded at a concentration of 2 million cells per 1.5 ml medium. The $CD34^+$ cells were seeded at 100.000 cells per ml medium in 2D as previously established for this type of culture [95, 97]. When deciding at which concentrations to seed $CD34^+$ cells into 3D culture we decided to use numbers enabling us to directly compare MNCs and $CD34^+$ cells in 3D cultures. Therefore, we calculated the percentage of $CD34^+$ cells in the MNC fraction used for separation of each individual sample and seeded the $CD34^+$ cells at numbers representing two million MNCs in each scaffold. This ranged from around 17.000 to 80.000 cells per 1.5 ml medium depending on the cell composition of each sample. The cells were cultured for four weeks where the medium was optimized for erythroid maturation with three different phases. For the first week the medium was supplemented with the serum substitute BIT9500, SCF, IL-3, and interleukin-6 (IL-6). From the second week EPO and iron saturated human transferrin were added to the medium. From the third to fourth week BIT9500, IL-3 and IL-6 were removed and replaced with fetal bovine serum (Figure 7).

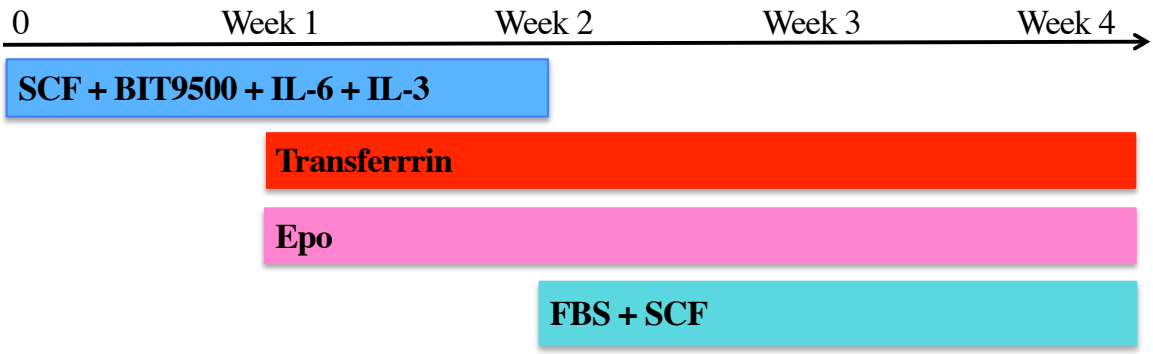


Figure 7 Medium composition throughout 4 weeks of culture. We used Iscove's Modified Dulbecco's Media (IMDM) supplemented with stem cell factor (SCF), BIT9500, interleukin 6 (IL-6), interleukin 3 (IL-3), transferrin, erythropoietin (EPO) and fetal bovine serum (FBS).

This composition was determined for the cells to be able to settle into culture without the stress of driving them towards erythropoiesis during the first week of culture, followed by support for erythroid maturation in subsequent weeks. Medium was changed every two to three days and cells collected at the end of week two, three and four for analysis.

3.2 SCAFFOLD FABRICATION AND STERILIZATION

Scaffolds were made via thermally induced phase separation followed by solvent sublimation, cutting and coating with collagen type I as previously described [78, 127]. This was followed by sterilization and washing before cell seeding. The scaffolds were designed by Mortera-Blanco and associates, who optimized the pore size, cell number concentrations and protein coating to be able to sustain long term cytokine free culture of AML cell lines and cord blood MNCs *in vitro* [78, 127]. Collagen can support the localization of myeloid and erythroid progenitors and therefore enables the cells to adhere and form niches within the scaffolds [128]. This prompted us to use these scaffolds to provide a structural component resembling the bone marrow architecture and allowing for cellular niche formations *in vitro*.

3.3 FLOW CYTOMETRY AND FACS

Flow cytometry and fluorescence-activated cell sorting (FACS) are techniques used to respectively analyze and isolate cells based on expression of surface proteins on the cells' exterior membrane. This is accomplished through staining of the cells with fluorophore-

conjugated antibodies after which the cells are interrogated by passing through the beam of a laser, exciting any fluorophores bound to the cells surface. In the studies included in this thesis we used a combination of the surface markers CD45, CD235a, the viability marker Aqua and the nuclear stain Draq5™ to determine the stage of erythropoiesis in our cultures. We defined non-erythroid hematopoietic cells as Aqua negative, CD45 positive and CD235a negative, erythroid progenitors as Aqua negative, CD45 negative, CD235a low and Draq5™ positive, intermediate erythroblasts as Aqua negative, CD45 negative, CD235a high and Draq5™ positive and enucleated erythrocytes as Aqua negative, CD45 negative, CD235a positive and Draq5™ negative. These populations were FACS sorted, spun onto slides and stained for morphological confirmation. To be able to detect viable enucleated erythroid cells we decided to use a viability marker based on membrane integrity (Aqua) in combination with the erythroid marker CD235a and a nuclear stain. Draq5™ unfortunately has a broad excitation and emission range which makes it difficult to include far-red fluorochromes, prompting us to keep our panel as simple as possible. For compensation purposes we used single stain and fluorescent minus one (FMO) controls [129] and the gating strategy was based on FMOs and biologically separate populations. Using this strategy, we sorted out the different populations from an MDS-RS patient in study **I** and were able to determine that ring sideroblasts are negative for CD45 and positive for CD235a (Figure 8).

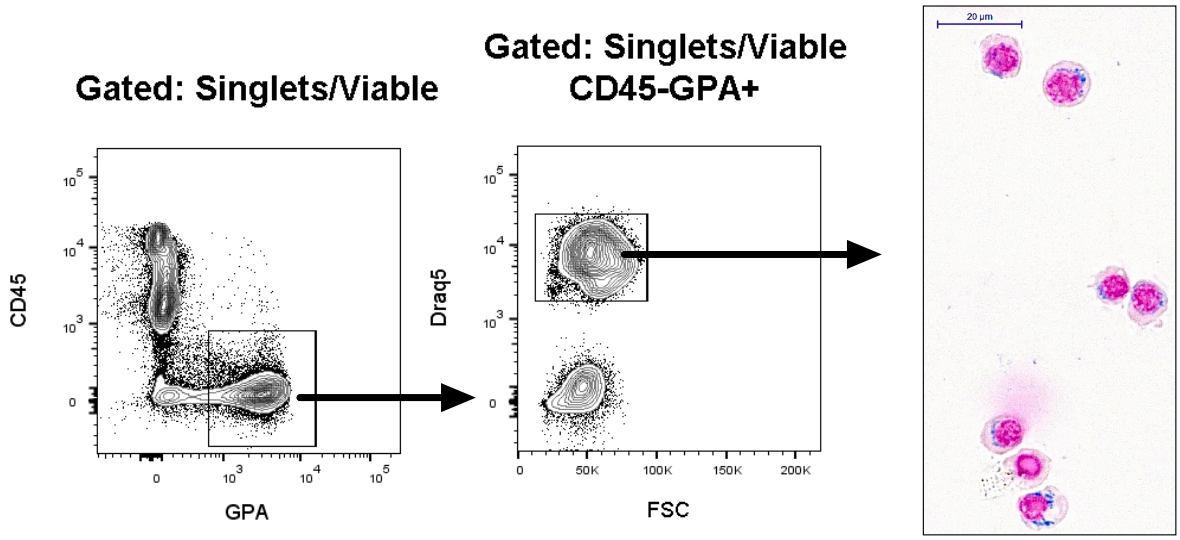


Figure 8 FACS gating strategy for selection of the population including ring sideroblasts (scale bar; 20 µm). The figure is adapted from Mortera-Blanco *et al* 2017 [130].

In studies **II** and **III** this strategy was used to define the different stages of erythropoiesis throughout culture. To avoid bias as a result of difficulties in extracting every cell from the scaffolds, we reported the size of each population as a percentage of the entire analyzed sample.

3.4 PROLIFERATION ASSAY

In order to measure cellular expansion throughout our cultures we used the one step aqueous soluble tetrazolium/formazan MTS assay. In this assay the tetrazolium compound is bio reduced into formazan by enzymes found in metabolically active cells and the quantity of formazan is then measured by absorbance at 490nm, which is proportional to the number of living cells in the culture [131]. We decided to use the MTS assay since we could add the compound directly to our cultures enabling measurement of proliferation without removing the cells from the scaffolds. This was done to avoid cellular stress and introducing potential bias as a result of some of the cells being left behind in the scaffolds.

3.5 FUNCTIONAL STEM AND PROGENITOR CELL ASSAYS

Long-term culture colony forming cell (LTC-CFC) assays can be used to functionally identify and quantify the most primitive hematopoietic progenitor cells with self-renewal capacity *in vitro* [132-135]. The assay is composed of a co-culture of hematopoietic progenitor cells on irradiated feeder cells for six weeks followed by two weeks of colony-forming unit cell (CFU-C) assays, enabling only primitive progenitor cells with self-renewal potential to survive and maintain their functional properties [134-136]. In our experiments we used the murine stromal cell lines M2-10B4 and Sl/Sl that have been engineered to express the human cytokines SCF, IL-3 and G-CSF [137]. Although LTC-CFC assays cannot measure the repopulation capacity of HSCs as in long-term transplantation assays, they can be used as a substitute when such experiments are not feasible.

In study **I** we used the LTC-CFC assay to assess the self-renewal potential of MDS-RS stem and progenitor cells compared to healthy controls, and to investigate the cellular origin of the *SF3B1* mutated clone. In study **II** we used the LTC-CFC assay to determine if cells with self-renewal potential were maintained throughout the four weeks of culture in our scaffold system. Since the LTC-CFC assay was developed for seeding hematopoietic progenitor cells we tried

to optimize well size, and numbers of stromal and seeded hematopoietic cells to be able to seed cultured MNCs in LTC-CFC assays. Unfortunately, the difference in cellular composition between individual samples of cultured MNCs resulted in such a variation in colony numbers that we decided to only record if they were present or not.

3.6 TARGETED SEQUENCING

Heterozygous *SF3B1* single nucleotide mutations were detected in our cohort of MDS-RS patients using targeted sequencing of DNA isolated from bulk bone marrow. For this purpose, Haloplex selector probes were used as previously described [98] or the TruSight myeloid sequencing panel (illumina®). Pyrosequencing and droplet digital PCR (ddPCR) were used to confirm the presence and quantify the allele burden or VAF of these previously identified mutations.

3.6.1 Pyrosequencing

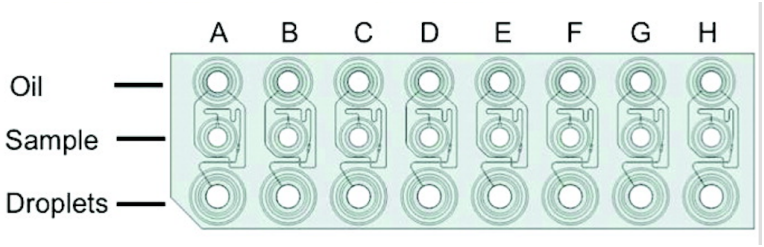
Pyrosequencing is a targeted DNA sequencing by synthesis method that can identify single bases or short stretches of nucleic acid sequence. This works via bioluminescence, where pyrophosphate release is converted to light through an enzymatic reaction during incorporation of a nucleotide into a growing chain of DNA [138]. This process involves the following steps: 1) A sequencing primer designed for the target of interest is added to a single strand DNA template and incubated with the enzymes DNA polymerase, adenosine triphosphate (ATP) sulfurylase, luciferase and apyrase along with the substrates adenosine 5´phosphosulfate and luciferin. 2) Deoxynucleotide triphosphates (dNTPs) are added one at a time and the DNA polymerase incorporates the complementary dNTP into the DNA template which then releases pyrophosphate (PPi). 3) In the presence of adenosine 5´phosphosulfate the ATP sulfurylase converts PPi to to ATP, which acts as a substrate for the luciferase-mediated conversion of luciferin to oxyluciferin, generating visible light in proportion to the amount of ATP. 4) The light produced is detected by a camera and analyzed in a computer program. 5) Unincorporated nucleotides and ATP are degraded by apyrase and the reaction starts again with another nucleotide. Water was run as a negative control for each reaction and *SF3B1* wild type DNA both from a healthy individual and from the patients themselves (DNA from isolated T-cells) were used as biological controls. In study **I** we used pyrosequencing to detect *SF3B1* mutations

in LTC-CFC and CFC colonies and in pro-B cells from MDS-RS patients. Since there was a risk of cross contamination between individual colonies we considered colonies to be positive with a VAF above 25% and negative with a VAF of 5% or lower (and those in between to be inconclusive). For the FACS sorted pro-B cells we used a 5% cutoff. In study **II** we also used pyrosequencing to evaluate if the *SF3B1* mutated clone was preserved throughout the different MDS-RS cultures with a 5% cutoff for positivity.

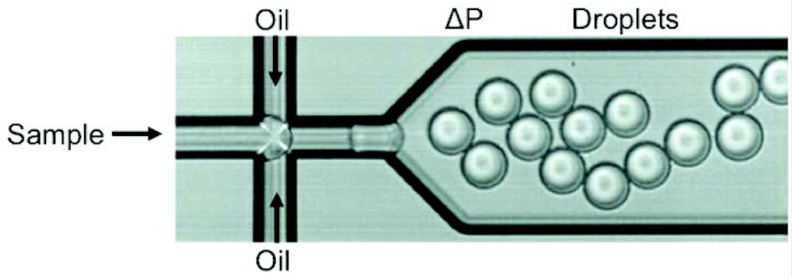
3.6.2 ddPCR

Since the sensitivity of pyrosequencing can vary between different cell types, and false negatives can occur after amplification of low amounts of DNA, we decided to move forward using a more sensitive method called ddPCR. This method was used to analyze cells from xenotransplantation experiments in study **I** and to follow the size of the *SF3B1* mutated clone throughout culture in study **III** (sensitivity set to 0.1%). Digital polymerase chain reaction (PCR) is a method where partitioning of individual molecules into many replicate reactions at limiting dilutions is used for absolute nucleic acid quantification [139]. In ddPCR, reactions are partitioned into thousands of highly uniform, nanoliter-sized, aqueous droplets in oil per sample, enabling a precise calculation of concentrations via Poisson correction for multiple target molecules per droplet [140]. This process includes the following steps: 1) DNA samples and targeted probes specific for the assayed mutation (one for the mutant allele and one for the wildtype allele) are loaded into a droplet generator cartridge together with droplet generation oil. 2) Vacuum is used to draw the sample and oil through a flow-focusing nozzle where in less than two minutes each sample is converted into 20.000 one nanoliter droplets. 3) After droplet generation the samples are pipetted into a PCR plate where PCR amplification is performed in a thermal cycler. 4) The plate is loaded onto a reader that absorbs the droplets and streams about 1000 of them per second in a single-file past a two-color detector. 5) The droplets are marked as positive or negative based on fluorescence. Their number in each channel is used to calculate the concentration of target and reference DNA sequences and their Poisson-based 95% confidence intervals (Figure 9) [140].

1. DNA sample and droplet generation oil loaded into cartridge

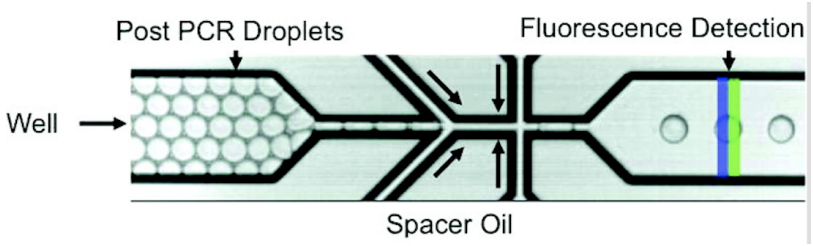


2. Droplets generated via vacuum



3. PCR amplification

4. Reader absorbs droplets and reads their fluorescence



5. Concentration of target and reference DNA sequence calculated

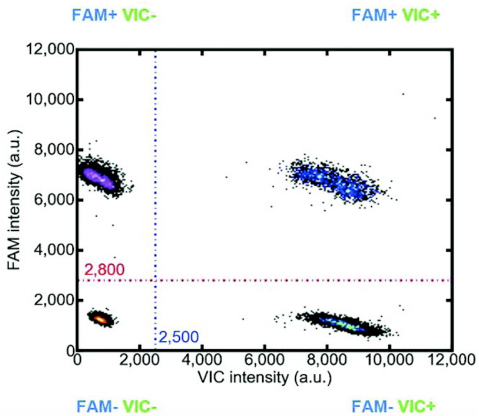


Figure 9 The different steps of the ddPCR workflow (see main text). The plot in stage 5 represents the measured wild type (HEX) and mutant (FAM) fluorescent signals for each droplet. The figure is adapted from Hindson *et al* 2011 [140].

At least a triplicate of DNA samples from healthy individuals were run for every probe/assay for validation and water was run as a negative control for each reaction.

3.7 MORPHOLOGICAL AND HISTOPATHOLOGICAL EVALUATIONS

Cells extracted from our cultures were spun onto microscope slides and either stained with May-Grünwald Giemsa or Perl´s Prussian blue for ring sideroblast detection using standard pathology procedures. The 3D scaffolds themselves are essentially like a sponge which we gently squeezed and flushed with buffer to recover as much of the cells out as possible. Aspirating all the cells out of the scaffolds proved to be difficult since some cells attached strongly to the collagen coated walls of the scaffolds, as could be expected. In study **II** we therefore decided to see if we could treat the scaffolds as bone marrow biopsies or solid tissue by fixing them in 4% paraformaldehyde overnight, and then either embedding them in paraffin or use cryopreservation. We quickly learned that the scaffolds would easily tare and lose their original structure after paraffin embedding (Figure 10A) but in some cases we managed to detect erythroid clusters and cells attached to the walls of the scaffolds. We stained paraffin sections from already aspirated scaffolds with Haemotoxylin and Eosin and Nestin and confirmed that there were some cells left lining the walls of the scaffolds (Figure 10B).

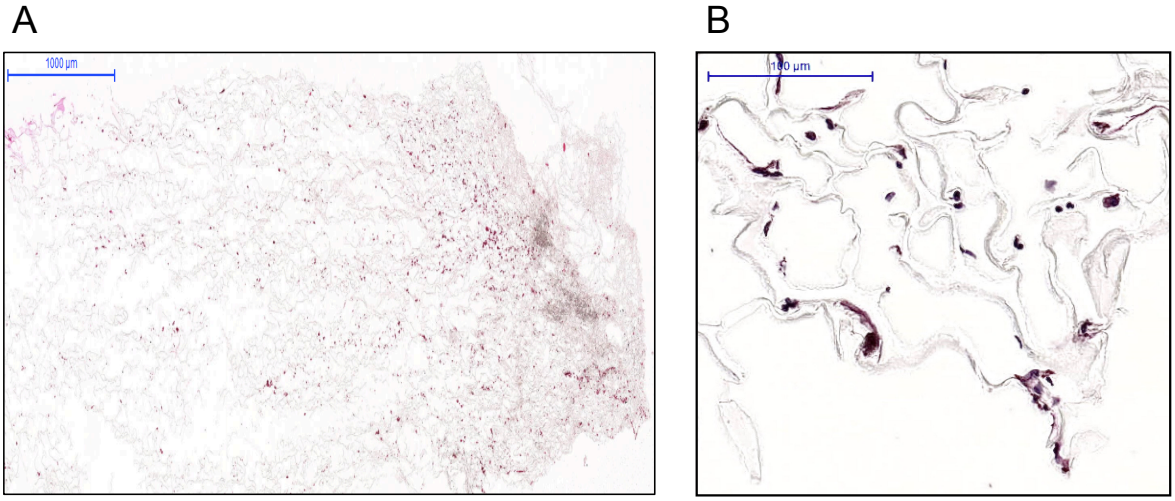


Figure 10 Paraffin embedded scaffold sections (A) from week 3 of healthy bone marrow culture stained with H&E (scale bar; 1000 µm) and (B) from week 4 of healthy bone marrow culture where scaffolds were aspirated before embedding and stained with Nestin (scale bar; 100 µm).

This we had to take into account when comparing the 2D and 3D cultures and prompted us to use an *in situ* proliferation assay (MTS) to minimize bias.

The cryopreserved scaffolds did not tear to the same extent as the paraffin embedded ones and cells were more easily retained within the scaffolds. We stained sections from cryopreserved scaffolds with fluorescently conjugated antibodies which enabled us to detect cellular clusters

and erythroblastic islands via confocal microscopy, although unfortunately the autofluorescence of the polyurethane scaffolds themselves introduced some limitations.

3.8 CYTOKINE MEASUREMENTS

To determine if there was a difference in cytokine secretion after 3D culture of MNCs vs $CD34^+$ cells, healthy bone marrow vs MDS-RS samples, and if this secretion was affected by luspatercept treatment, we froze medium from week one, two and four (representing change in medium composition) from our different cultures in studies **II** and **III**. For this purpose, we measured GDF11 with a sandwich enzyme linked immune-sorbent assay (ELISA) and used a commercially available bead-based multiplex assay that uses the Luminex® technology to measure concentration of 19 cytokines that were of interest and available. Those were TGFα, G-CSF, Flt-3L, GM-CSF, IFNγ, IL-10, MCP-3, IL-13, IL17A, IL-1a, IL-9, IL-6, IL-8, MCP-1, MIP-1a, MIP-1β, TNFα, TNFβ and VEGF. Standard curves made from measuring recombinant human cytokines of known concentrations were used to convert absorbance or fluorescent units to cytokine concentration units. The medium was frozen immediately after extraction at -80°C and only thawed once directly before usage to minimize degradation [141]. Supernatants from empty scaffolds treated identically to scaffolds seeded with cells were used as controls.

4 RESULTS AND DISCUSSIONS

4.1 STUDY I

Although it was well-known that the majority of MDS-RS patients harbor recurrent somatic mutations in *SF3B1,* resulting in a negative impact on erythropoiesis, the primary target of these mutations and at which stage of hematopoietic hierarchy the mutated clone gains competitive advantage had not been well established at the time of publication of study **I**. It had been implied that *SF3B1* mutations target the phenotypic HSCs compartment [142], although the lymphoid lineages were thought to not be involved, giving support to the possibility that downstream myeloid progenitors could be the cell of origin in MDS-RS [88, 142, 143].

4.1.1 Investigation

To investigate if there were differences in the frequency of stem and progenitor cell compartments in MDS-RS compared to healthy individuals, HSCs, CMP, GMP and MEPs were FACS sorted as previously described [90] with no significant differences detected. These populations were further seeded into CFC assays revealing a reduced capacity of GMPs and MEPs to generate myeloid and erythroid colonies, with poor hemoglobinization of BFU-Es (Figure 1D, Study **I**). The LTC-CFC assay was used to investigate the self-renewal potential of MDS-RS stem and progenitor cells, revealing it to be restricted to the HSC compartment and significantly reduced compared to healthy individuals. Next, we used computational predictions based on targeted sequencing data to evaluate the order of mutations in patients with more than one recurrent driver mutations, showing that *SF3B1* mutations were predicted to be the first event in most cases. Individual colonies from LTC-CFC assays seeded with FACS sorted HSCs and directly FACS sorted HSCs, GMPs and MEPs from MDS-RS patients were all found to be *SF3B1* mutated while T-cells were negative. To further investigate the lymphoid compartment, FACS sorted pro-B cells from MDS-RS patients were found to be reduced compared to healthy controls and to harbor *SF3B1* mutations, while mature B cells were wildtype in all but one case. To assess the propagating ability of MDS-RS stem and progenitor cells *in vivo* we transplanted FACS sorted HSCs, CMPs, GMPs and MEPs from two patients into immunodeficient mice and analyzed after 20 to 22 weeks. We found that only the HSCs sustained myeloid and B-cell engraftment with clonal involvement of myeloid cells

generated from one of the engrafted patients. Furthermore, we showed that ring sideroblasts are exclusively CD45 negative and CD235a positive and found ring sideroblasts via immunohistochemistry in all mice transplanted with HSCs from *SF3B1* mutated MDS-RS patients.

4.1.2 Key findings

In this study we show that the self-renewal capacity of MDS-RS stem and progenitor cells is significantly reduced and restricted to the primitive HSC compartment, and that only these HSCs can propagate the *SF3B1* mutated clone both *in vitro* and *in vivo.* These results indicate that HSCs from MDS-RS patients do not pass on self-renewal potential to downstream progenitors. Importantly we found *SF3B1* mutations in the pro-B cell compartment of MDS-RS patients, thereby proving that recurrent *SF3B1* mutations target the multipotent lymphomyeloid HSCs and have a negative impact on lymphoid development. We did not find clonal involvement in the T-lymphoid lineage, which might be a reflection of lymphoid development being incompatible with *SF3B1* mutations, or of the longevity of T-cells which are not actively produced in the elderly [5, 144, 145] (Figure 11).

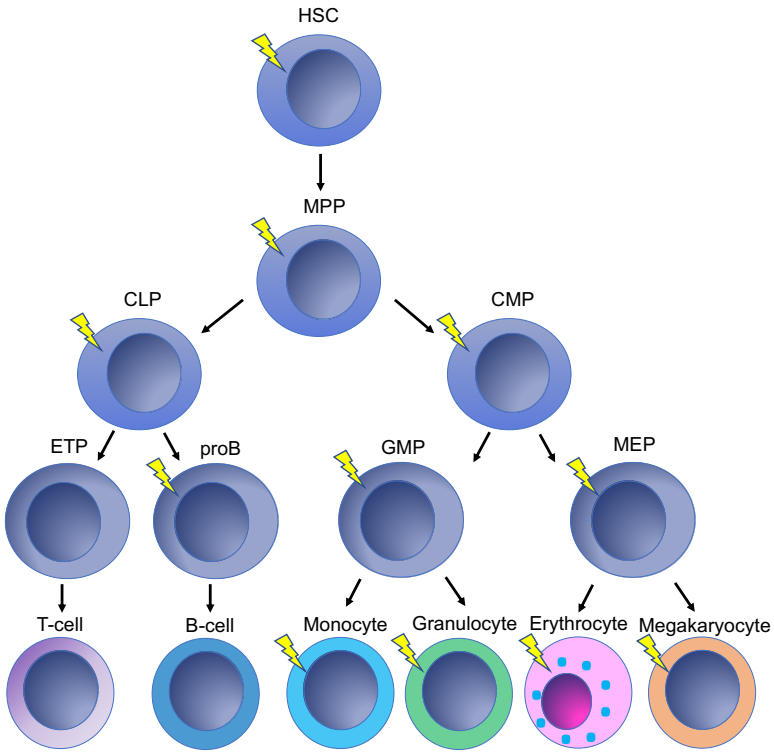


Figure 11 Origin of *SF3B1* mutations in MDS-RS. We showed that the *SF3B1* mutations originate before division into lymphoid and myeloid lineages. Lightning represent *SF3B1* mutation. Hematopoietic stem cell (HSC), multipotent progenitor (MPP), common lymphoid progenitor (CLP), early T-cell progenitor (ETP), B-cell progenitor (proB) granulocyte/macrophage progenitor (GMP) and megakaryocyte/erythroid progenitor (MEP). The figure is adapted from Meyer 2017 [7].

Previously it had been reported that no ring sideroblasts were obtained after transplantation of HSCs from MDS-RS patients into immunodeficient mice [142]. In that study expression of CD45 was used to determine engraftment, which would have excluded detection of ring sideroblasts according to our observation that they are CD45 negative. We therefore investigated the whole bone marrow of transplanted mice and reported for the first-time ring sideroblast generation in immunodeficient mice after transplantation with HSCs from MDS-RS patients, providing a new methodology to study the dysregulated erythroid development in MDS-RS patients.

4.2 STUDY II

Modelling ineffective erythropoiesis *in vitro* has thus far been a challenge, limiting our capacity to functionally study the regulatory mechanisms involved and to screen for novel therapeutic

compounds. Primary suspension cultures of erythroid progenitors from MDS-RS patients have been useful to study the early stages of erythropoiesis, with increased mitochondrial ferritin and mitochondria mediated apoptosis observed [95-97]. Unfortunately cultures capable of consistently generating ring sideroblasts, erythroblastic islands and mature erythrocytes have not been reported, which would be required to further elucidate the biological consequences of *SF3B1* mutations. Although xenograft models are a valuable model for studying MDS-RS erythropoiesis, low engraftment and recovery of MDS-RS cells limit the capacity for experimental outputs [146, 147]. In this study we therefore wanted to establish an *in vitro* model capable of recapitulating both healthy and MDS-RS erythropoiesis with formation of ring sideroblasts and maintenance of the *SF3B1* mutated clone.

4.2.1 Investigation

According to our results in study **I,** ring sideroblasts could be generated when HSCs from MDS-RS patients were provided with the bone marrow microenvironment of the mouse. Additionally, including autologous mesenchymal stromal cells was reported to facilitated engraftment of MDS hematopoiesis in mice [148]. We therefore decided to culture the whole MNC fraction (including stromal cells) or isolated $CD34^+$ cells from the bone marrow of healthy individuals and MDS-RS patients, either in suspension (2D) or in polyurethane scaffolds (3D). Using flow cytometry, we found that both 3D and 2D culture of MNCs from healthy individuals primarily facilitated culture of non-erythroid hematopoietic cells, although the percentage of erythroid cells produced was slightly higher in 3D than in 2D cultures. Interestingly, while $CD34^+$ cells did not survive four weeks of 2D culture the opposite was observed in 3D, where they also generated significantly higher amounts of erythroid cells compared to 3D and 2D MNC cultures. Although less pronounced than in healthy individuals, erythropoiesis was also favoured in 3D culture of $CD34^+$ cells from MDS-RS patients. The $CD34^+$ 3D cultures additionally had the highest proliferative capacity both in healthy and MDS-RS cultures, while they completely stopped proliferating after three weeks in 2D culture. MNC cultures retained stable proliferation throughout the culture period. We then used LTC-CFC assays to demonstrate that stem and progenitor cells with self-renewal capacity were maintained after four weeks in the $CD34^+$ 3D, MNC 3D and MNC 2D cultures. We also found erythroblastic islands in all three culture types after four weeks of healthy and MDS-RS cultures. To detect if cells cultured in 3D secreted factors at different concentrations between

MNC and CD34$^+$ cultures, and between healthy and MDS-RS cultures, we measured secretion of selected cytokines. We found that while 3D CD34$^+$ cultures produced low levels of cytokines in general, TGF-β1 - a factor reported to have a negative effect on erythropoiesis and a sustained signal activation in MDS [149-153] - was secreted at higher levels after four weeks of MDS-RS compared to NBM cultures. GDF11 was only detected in 3D culture of MNCs from MDS-RS patients, while secretion of IL-10 was increased and IL-1α secretion lost throughout MNC cultures, reflecting erythroid differentiation [20, 154]. Importantly pyrosequencing revealed the maintenance of *SF3B1* mutated clones throughout CD34$^+$ 3D, MNC 3D and MNC 2D cultures with regeneration of ring sideroblasts from the second week of culture.

4.2.2 Key findings

In this study we report for the first time that 3D culture of primary CD34$^+$ cells from healthy individuals and MDS-RS patients enables long-term continuous cellular expansion and facilitates erythropoiesis, including generation of erythroblastic islands and enucleated red cells. Corresponding MNC cultures could also be maintained long-term although with lower proliferative capacity and mostly facilitating the expansion of non-erythroid hematopoietic cells. This is in line with reports showing that co-culture of CD34$^+$ cells with stromal cells, or with conditioned media produced by stromal cells, has a negative effect on erythropoiesis in favor of the myeloid lineage [46, 126]. The different capability of the CD34$^+$ cells to survive and proliferate in 2D and 3D might be explained by the structural component provided by the scaffolds, where the cells have the opportunity to form cellular niches allowing for cell-cell and cell-matrix interactions similar to *in vivo*. We also demonstrated that MNCs and CD34$^+$ cells cultured in 3D retained self-renewal capacity throughout culture and generated erythroblastic islands which are an essential component for erythropoiesis [39, 40, 155]. Importantly, the MDS-RS cultures maintained the *SF3B1* mutated clone and consistently generated ring sideroblasts. To our knowledge, this is the first report of successful *de novo* generation of ring sideroblasts *in vitro.* These results support the usage of the CD34$^+$ 3D culture for studying healthy and MDS-RS erythropoiesis in a simple and effective way where it is easier to control for cellular input and recovery compared to MNC cultures. The MNC cultures on the other hand facilitated secretion of cytokines known to have an effect on erythropoiesis and might therefore be an alternative model for studying effects of stroma or other non-erythroid

hematopoietic cells on erythropoiesis. This model can be used to study physical, molecular and cellular components important for erythropoiesis and as an alternative to animal models for high-throughput screening of therapeutic compounds aiming to alleviate anemia in patients.

4.3 STUDY III

While the TGFβ superfamily ligand trap luspatercept shows promising results in patients with MDS-RS, it still remains unclear if the drug supports terminal erythroid differentiation of the *SF3B1* mutated disease clone or if it gives the wild type clone a competitive advantage in the bone marrow of patients. Recent reports also question if the drug works primarily through binding of GDF11 [125] and suspension cultures have not recapitulated the erythroid enhancing effect of the drug [22]. This lead us to investigate possible pathways of drug effect and if there may exist a secondary effect through MDS-RS stroma.

4.3.1 Investigation

We cultured MNCs and $CD34^+$ cells from healthy individuals and MDS-RS patients in the 3D culture model established in study **II** and treated them with luspatercept from the second week of culture. To determine if luspatercept has a direct or secondary effect on erythropoiesis we compared proliferative capacity via the MTS assay and erythropoiesis via flow cytometry in cultures from healthy individuals. We found that luspatercept gave a non-significant but consistent increase in proliferation and erythroid cell production both for MNC and $CD34^+$ cells in 3D culture. In MDS-RS cultures, however, luspatercept did not show an effect on proliferation or erythropoiesis. We then measured the VAF of the patient-specific *SF3B1* mutations and counted ring sideroblasts after four weeks of MDS-RS cultures. In the three patients studied, no effect was observed after luspatercept treatment. To evaluate if cytokine secretion differed between MNC and $CD34^+$ cultures, and between normal BM and MDS-RS cultures, and if luspatercept treatment affected this secretion, we measured the concentration of cytokines in media extracted from cultures. We found that cytokines involved in erythropoiesis; granulocyte-macrophage colony-stimulating factor (GM-CSF), G-CSF, TNFα and fms like tyrosine kinase 3 ligand (Flt-3L or CD135) were secreted at higher concentrations at the beginning of MNC compared to $CD34^+$ cultures, but that these levels evened out throughout the culture period [32, 156-158]. Interestingly monocyte chemoattractant protein 1

(MCP-1 or CCL2), a cytokine highly expressed by erythroblastic island macrophages [159], followed the same trend although at much higher concentrations. This perhaps represents the formation of erythroblastic islands in the $CD34^+$ cultures. Vascular endothelial growth factor (VEGF), known to be expressed at increased levels in low-risk MDS [160], was secreted at significantly higher concentrations in MDS-RS compared to healthy MNC cultures. On the other hand, monocyte chemoattractant protein 3 (MCP-3 or CCL7) was secreted at higher, although not significantly, concentrations in healthy cultures. None of these cytokines were affected by luspatercept treatment. One of the measured cytokines, IL-6, was secreted at significantly higher concentrations during the second week of healthy $CD34^+$ cultures compared to all other culture types. Interestingly this secretion was completely lost when the cells were treated with luspatercept. To see if there was a difference in IL-6 receptor expression on healthy compared to MDS-RS $CD34^+$ cells we searched previously reported RNA sequencing genome data [161] and found it to be significantly lower in MDS-RS.

4.3.2 Key findings

In this study we report that luspatercept enhances proliferation and drives both MNCs and $CD34^+$ cells from healthy individuals towards erythropoiesis, indicating that the drug has a direct effect on erythroid progenitors in 3D culture. With respect of the hitherto limited observations, we did not see the same effect in luspatercept treated MDS-RS cultures, nor could we find differences in *SF3B1* mutated clone size or ring sideroblast percentage following exposure. These results imply that luspatercept does not directly inhibit the disease clone, although we cannot exclude that luspatercept might act by reducing erythroid apoptosis or that we hitherto may have included only non-responders in our study. Therefore, we will address these uncertainties in a larger cohort of patients. We also demonstrated that luspatercept may have an effect on cytokine secretion by showing that the drug completely inhibited the secretion of IL-6 from $CD34^+$ cultures of healthy bone marrow. There are contradicting reports with regards to expression levels of IL-6 in MDS patients [162-164], although this might be the result of pooling measurements from different MDS subgroups. We therefore demonstrated that the expression of the IL-6 receptor is downregulated on $CD34^+$ cells from MDS-RS patients. Interestingly, IL-6 has been found to impair mitochondrial function in maturing erythroid cells *in vitro* and to have a negative effect on erythroid cells through capsase-3 activation and apoptosis [49, 165]. Only 25% of MDS patients are reported to have elevated

levels of IL-6 [166] and 37.9% of MDS-RS patients achieve transfusion independence after luspatercept treatment [123]. Therefore, it may be worth exploring if luspatercept responders have elevated levels of IL-6 compared to non-responders.

5 CONCLUDING REMARKS AND FUTURE OUTLOOK

More than 80% of patient with MDS-RS harbor recurrent somatic mutations in the splicing factor gene *SF3B1* [98] where the downstream ramifications on erythropoiesis have yet to be elucidated. In study **I** we demonstrated that only the HSCs from MDS-RS patients could propagate and sustain the *SF3B1* mutated clone *in vitro* and *in vivo,* and that the clone originates in the multipotent lymphomyeloid HSCs. We provided definite evidence that *SF3B1* mutations were present at the pro-B cell stage and have a negative effect on lymphoid development. These results are particularly interesting in light of recent publications where age related inflammation of the bone marrow microenvironment has been shown to induces ineffective erythropoiesis [49]. Furthermore, elevated levels of damage associated molecular patterns S1008/S1009 have been found in the serum of MDS patients, creating an immunosuppressive microenvironment [167]. We also demonstrated that transplanting immunodeficient mice with HSCs from MDS-RS patients resulted in ring sideroblast generation in the mouse bone marrow in all cases. Interestingly, genetic mouse models allowing conditional expression of *SF3B1* mutations have not been reported to generate ring sideroblasts [110, 111]. This might result from the lack of additional mutation found to co-occur with *SF3B1* mutations in MDS-RS patients, or from unresolved differences in mitochondrial iron metabolism between human and mice [110]. Recently formation of ring sideroblasts has been reported in a cytokine-humanized MDS patient derived xenotransplantation model, circumventing the downstream differences between mice and man [168]. The MDS xenotransplantation model represents a novel *in vivo* platform for exploring the cellular and molecular basis as well as therapeutic targets in MDS-RS.

In study **II** we established a 3D *in vitro* culture system using primary cells from MDS-RS patients, capable of mimicking dysregulated erythropoiesis including formation of ring sideroblasts and maintenance of the *SF3B1* mutated disease clone. This model provides the opportunity to study the physical, molecular and cellular components involved in terminal erythropoiesis. It could also be a valuable method for high-throughput screening of drugs aimed to alleviate anemia in patients in an easier and cheaper way than using mouse models. Producing hiPSCs that recapitulate different combinations of mutations found to co-occur with *SF3B1* mutations in MDS-RS patients and to establish MDS-RS derived cell lines capable of producing ring sideroblasts could be the next important step in modelling the disease. Recently some ring sideroblast generation has been reported from hiPSC cultures derived from an MDS-RS patient, but only after genetic manipulation to induce expansion of hematopoietic

progenitor cells [106]. Since $CD34^+$ hematopoietic progenitor cells grew extremely well while maintaining their self-renewal potential in our culture system, using the 3D scaffolds to culture MDS-RS derived hiPSCs might help to overcome the limited hematopoietic potential of MDS hiPSCs. Additionally, the system might be of value to study stromal effect on erythropoiesis via co-culture of healthy stroma with MDS hematopoietic cells, and vice versa, and to model other erythroid failure disorders.

In study **III** we tested the effect of TGFβ superfamily ligand trap luspatercept on MNCs and $CD34^+$ cells from healthy individuals and MDS-RS patients cultured in the 3D model established in study **II**. We found an increase in proliferation and erythropoiesis in cultures from healthy individuals treated with luspatercept, while similar effects were not detected in MDS-RS cultures. Moreover, the *SF3B1* mutated clone size and ring sideroblast percentage was not affected by treatment. It is possible that the drug allows for terminal maturation of erythroid cells from the disease clone, although we cannot exclude the possibility that luspatercept might reduce erythroid apoptosis. This will have to be further investigated in a larger cohort of MDS-RS patients, preferentially known to respond or not to luspatercept treatment. With regard to cytokine secretion, we found that IL-6 secretion by healthy $CD34^+$ cells in 3D culture was completely prevented with luspatercept treatment. As previously mentioned ring sideroblasts have iron filled mitochondria inducing caspase-9 activation, cytochrome *c* release and apoptosis [95]. Interestingly IL-6 impairs mitochondrial function in maturing erythroid cells *in vitro* [165] and reduces erythroid colony formation through caspase-3 activation and apoptosis *in vivo* [49]. Further investigation into IL-6 secretion and its effect on MDS-RS erythropoiesis might therefore be of interest. Additionally, only 25% of MDS patients are reported to have elevated levels of IL-6 [166]. We therefore plan to explore cytokine patterns in MDS-RS patients *in vitro* with different clinical outcome after luspatercept treatment.

6 ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to everyone who contributed and supported me throughout my time as a PhD student, in particular:

My supervisor **Eva Hellström-Lindberg** for giving me this opportunity, encouraging my curiosity and pushing me forward when I needed it. I feel lucky to have been supervised by someone who is not only an inspirational researcher but also genuinely loves science and wants her students to succeed.

To my co-supervisors; **Iyadh Douagi** thank you for all your advice, scientific and personal, you have both been a supervisor and a mentor to me. And thank you **Petter Woll** for taking me on as a student and spending time on teaching me, I am a better scientist for it.

I also want to thank **Sten-Eirik Jacobsen** for all of his advice during his time as my co-supervisor and **Birgitta Sander** for her kindness and the time she spent on my projects and teaching me about pathology.

Thank you to all the PIs who are/where at HERM, it has been inspiring to work in an environment where all the group leaders are willing to give their advice to any student that asks for it. **Evren Alici** I really appreciated the times we talked, that you took time to give me an interview and the input you gave me on my science. **Hong Qian** your input on the microenvironment was invaluable. **Julian Walfridsson** and **Robert Månsson** thank you for your scientific advice and fun conversations over lunch. **Johanna Ungerstedt** and **Martin Jädersten,** your clinical input and social discussions at various conferences and meetings were highly appreciated. Thank you **Petter Höglund** for great scientific input at seminars and for the work you do as a study director. **Seishi Ogawa** I am grateful for your scientific advice and cheerful presence. **Mattias Carlsten**, **Vanessa Lundin, Sidinh Luc, Yenan Bryceson** and **Sören Lehman** thank you for contributing to the friendly environment at HERM.

To my current and former group members; First of all, thank you **Monika Jansson** for always helping everyone in whichever way you can and for being so patient and kind. HERM would not have been the same without you. **Gunilla Walldin** thank you for your help with the patient samples and for always being up for a laugh. **Annette Öster Fernström, Ann-Charlotte Björklund, Ashma Ben Azenkoud, Indira Barbosa** and **Sara von Bahr Grebäck** thank you for your hard work and all the help throughout the years. **Mohsen Karimi**, **Christian Scharenberg** and **Magnus Tobiasson** I am grateful for your inspiration and advice. **Leonie Saft** your help with the pathology was greatly appreciated. **Mahin Nikougoftar Zarif** thank you for all the hard work you put into our project. **David Chang**, it was great fun having your company in the lab. **Simona Conte** thank you for welcoming me as a fellow PhD in the group, there was never a dull moment with you there. **Maria Creignou**, it was great fun to get to know you and I appreciate all your hard work on our project. **Isabel Hofman** thank you for all the

advice and help, you gave me the last push needed to finish this. **Marios Dimitriou** thank you for your endless patience, you have been an incredible teacher and I would not be half the scientist I am without your guidance. **Teresa Mortera-Blanco** not only have you taught me so much scientifically but you have also been an amazing friend, I feel so grateful and lucky to have met you.

I also want to thank all my current and former HERM fellows. **Michael Chrobok** it has been great to share this entire process with you, I am grateful to have made such good friends in you and your lovely wife **Sabrina Chrobok**. To my dear friend **Ying Qu**, you made my time at work so much more fun, thank you for always being there for me. **Caroline Gavin**, thank you for sharing the ups and downs of being a PhD student with me and for the fun times outside of work. **Carin Dahlberg**, I have really appreciated your advice and support, you are a great friend. Thank you **Huthayfa Mujahed, Monika Dolinska, Aditya Harisankar** and **Ayla De Paepe** for being my friends throughout this time, in spite of my sarcastic humor and never making it to after-work. To my former office mates, **Erle Refsum** you are really missed, **Srinivasa Rao Nagubothu** thanks for all the fun discussions and **Thibault Bouderlique** thank you for teaching me about microscopy and for the humor you bring to every situation. **Stephan Meinke** I really appreciate your patience, not getting tired of me asking where everything is when I first came to the lab. **Thuy Luu Thanh**, **Nadir Kadri, Yaser Heshmati** and **Sridharan Ganesan** thank you for bringing such fun energy to the lab. **Stina Virding** and **Desmond Chin**, thank you for all the fun times at retreats and your scientific input during meetings. **Anton Törnqvist, Charlotte Gustafsson**, **Cecilia Karlström**, **Jenny Rydén, Giovanna Perinetti Casoni, Heinrich Schlums, Caroline Leijonhufvud, Yaser Heshmati, Karin Belander-Strålin** and **Lucía Peña Pérez** thank you for your contributing to the friendly atmosphere at HERM. Thank you, **Hani Abdulkadir** and **Deepika Nair** for being so welcoming. Thank you to all the BMAs and permanent staff, you truly are the backbone of HERM. And to everyone else at HERM, thank you for making this a wonderful scientific and social environment, I will truly miss you all.

I am also grateful to the Department of Medicine, especially **Jan Bolinder**, **Anastasia Urban**, **Therese Lind**, **Elenor Nyman**, **Klas Karlsson** and **Ulrika Markne** for quickly and efficiently helping with any bureaucracy.

Importantly, I would also like to thank all the medical staff and patients for their invaluable contributions.

To my people in Sweden who made the decision to stay for a PhD so much easier:

Olof, Annika, Enrique, Aranza, Spiros and **Belen** you were the first friends I made in Sweden and I can´t thank you enough for all of your support, through thick and thin, during

this time. **Marie, Eleonor, Makz, Fredrik, Jesper** and **Malin** thank you for including me in your gang of misfits, teaching me Swedish and listening to my complaints no matter what. **Martin, Moses** and **Jonna** you have been instrumental in giving me the encouragement I needed to finish this chapter of my life, I am so lucky to have you. **Svanhildur, Jenny, Anna** and **Ida** thank you for helping me keep my sanity throughout the maternity leave, it gave me the energy I needed to get back to work and push through. And **Heiður**, I am so happy you decided to join us in Sweden, thank you for your encouragement and friendship. The **Brekkan´s** in Uppsala; thank you so much for including me as a part of your family, I really appreciate all of our time together.

To my friends at home:

I am so grateful for the endless support, encouragement and interest I have gotten from my wonderful friends in Iceland. **Kristína, Helga, Vala, Alda, Björgheiður, Ásdís, Stefanía, Unnsteinn** and **Helgi Freyr** thank you for believing in me, inspiring me and not giving up on me for being so bad at communicating from Sweden. You are such an empowering group of people.

To my family:

Unnsteinn, Agatha, Ylfa and **Elvar** the time you spent in Stockholm is very dear to me, thank you for cheering me on and giving me refuge when the trains got stuck. **Hugrún**, thank you for always showing interest in what I am doing and being an amazing sister and friend. **Estrid**, you are the best mother in-law one could get, your advice and help have been invaluable. **Mom** and **dad**, none of this would have happened if you hadn't taught me I could do anything I wanted with my life. Having parents who support me no matter what has been a tremendous privilege, and your encouragement is something I value deeply. To my little family; **Ari** thank you for believing in me and supporting me through the ups and downs, sharing the journey of a PhD with my partner has been incredible, me and Björk are very lucky to have you. To my daughter **Björk**, thank you for reviving my energy and ambition in life, I hope to inspire you to follow your dreams.

I would like to acknowledge the Karolinska Institutet, The Swedish Cancer Society (Cancerfonden), The Cancer Research Foundations of Radiumhemmet (Radiumhemmets Forskningsfonder), The Knut and Alice Wallenberg Foundation, The Government Public Health (ALF) Grants and the Swedish Research Council for their generous financial support.

7 REFERENCES

1. Spangrude, G.J., S. Heimfeld, and I.L. Weissman, *Purffication and Characterization of Mouse Hematopoietic Stem Cells.* Science, 1988. **241**(4861): p. 58-62.
2. Ogawa, M., et al., *Expression and function of c-kit in hemopoietic progenitor cells.* J Exp Med, 1991. **174**(1): p. 63-71.
3. Morrison, S.J. and I.L. Weissman, *The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype.* Immunity, 1994. **1**(8): p. 661-673.
4. Morrison, S.J., et al., *Identification of a lineage of multipotent hematopoietic progenitors.* Development, 1997. **124**(10): p. 1929-1939.
5. Kondo, M., I.L. Weissman, and K. Akashi, *Identification of clonogenic common lymphoid progenitors in mouse bone marrow.* Cell, 1997. **91**(5): p. 661-672.
6. Akashi, K., et al., *A clonogenic commonmyeloid progenitor that gives rise to allmyeloid lineages.* Nature, 2000. **404**(6774): p. 193-197.
7. Meyer, S.E., *Splicing together the origins of MDS-RS.* Blood, 2017. **130**: p. 841-842.
8. Rodriguez-Fraticelli, A.E., et al., *Clonal analysis of lineage fate in native haematopoiesis.* Nature, 2018. **553**(7687).
9. Carrelha, J., et al., *Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells.* Nature, 2018. **554**(7690): p. 106-111.
10. Karamitros, D., et al., *Single-cell analysis reveals the continuum of human lympho-myeloid progenitor cells.* Nat Immunol, 2018. **19**(1): p. 85-97.
11. Velten, L., et al., *Human haematopoietic stem cell lineage commitment is a continuous process.* Nat Cell Biol, 2017. **19**(4): p. 271-281.
12. Macaulay, I.C., et al., *Single-Cell RNA-Sequencing Reveals a Continuous Spectrum of Differentiation in Hematopoietic Cells.* Cell Rep, 2016. **14**(4): p. 966-977.
13. Crisan, M. and E. Dzierzak, *The many faces of hematopoietic stem cell heterogeneity.* Development, 2017. **143**(24): p. 4571-4581.
14. Ponka, P. and A. Sheftel, *Erythroid Iron Metabolism.* Iron Physiology and Pathophysiology in Humans, Nutrition and Health, 2012: p. 191-209.
15. Nandakumar, S.K., J.C. Ulirsch, and V.G. Sankaran, *Advances in Understanding Erythropoiesis: Evolving Perspectives.* Br J Haematol, 2016. **173**(2): p. 206-218.
16. Camaschella, C., et al., *The mutual control of iron and erythropoiesis.* Int J Lab Hematol, 2016. **38**: p. 20-26.
17. Vlahakos, D.V., K.P. Marathias, and N.E. Madias, *The role of the renin-angiotensin system in the regulation of erythropoiesis.* Am J Kidney Dis, 2010. **56**(3): p. 558-565.

18. Miyagawa, S., et al., *Insulin and insulin-like growth factor I support the proliferation of erythroid progenitor cells in bone marrow through the sharing of receptors.* Br J Haematol, 2000. **109**(3): p. 555-562.

19. Maguer-Satta, V., et al., *Regulation of human erythropoiesis by activin A, BMP2, and BMP4, members of the TGFβ family.* Exp Cell Res., 2003. **282**(2): p. 110-120.

20. Wang, C., W.K. Udupa, and D.A. Lipschitz, *Evidence Suggesting a Stimulatory Role for Interleukin-10 in Erythropoiesis In Vitro.* Journal of Cellular Physiology, 1996. **166**: p. 305-310.

21. Dussiot, M., et al., *An activin receptor IIA ligand trap corrects ineffective erythropoiesis in beta-thalassemia.* Nat Med, 2014. **20**(4): p. 398-407.

22. Suragani, R.N., et al., *Transforming growth factor-beta superfamily ligand trap ACE-536 corrects anemia by promoting late-stage erythropoiesis.* Nat Med, 2014. **20**(4): p. 408-14.

23. Tsai, F.Y., et al., *An early haematopoietic defect in mice lacking the transcription factor GATA-2.* Nature, 1994. **371**(6494): p. 221-226.

24. Rodrigues, N.P., et al., *Haploinsufficiency of GATA-2 perturbs adult hematopoietic stem-cell homeostasis.* Blood, 2005. **106**(2): p. 477-484.

25. Takahashi, S., et al., *GATA factor transgenes under GATA-1 locus control rescue germline GATA-1 mutant deficiencies.* Blood, 2000. **96**(3): p. 910-906.

26. Takahashi, S., et al., *Arrest in primitive erythroid cell development caused by promoter-specific disruption of the GATA-1 gene.* J Niol Chem, 1997. **272**(19): p. 12611-12615.

27. Fujiwara, Y., et al., *Arrested development of embryonic red cell precursors in mouse embryos lacking transcription factor GATA-1.* Proc Nati Acad Sci U S A., 1996. **93**(22): p. 12355-12358.

28. Hattangadi, S.M., et al., *From stem cell to red cell: regulation of erythropoiesis at multiple levels by multiple proteins, RNAs, and chromatin modifications.* Blood, 2011. **118**(24): p. 6258-68.

29. Li, J., et al., *Isolation and transcriptome analyses of human erythroid progenitors: BFU-E and CFU-E.* Blood, 2014. **124**(24): p. 3636-3645.

30. Fajtova, M., et al., *Immunophenotypic profile of nucleated erythroid progenitors during maturation in regenerating bone marrow.* Leukemia and Lymphoma, 2013. **54**(11): p. 2523-2530.

31. Mori, Y., et al., *Prospective isolation of human erythroid lineage-committed progenitors.* Proc Natl Acad Sci U S A, 2015. **112**(31): p. 9638-9643.

32. Chen, T.-L., et al., *Different effects of granulocyte colony-stimulating factor and erythropoietin on erythropoiesis.* Stem Cell Research and Therapy, 2018. **9**(119): p. 1-9.

33. Liu, J., et al., *Quantitative analysis of murine terminal erythroid differentiation in vivo: novel method to study normal and disordered erythropoiesis.* Blood, 2013. **121**(8): p. e43-e49.

34. Falchi, M., et al., *Dexamethasone targeted directly to macrophages induces macrophage niches that promote erythroid expansion.* Haematologica, 2015. **100**(2): p. 178-187.

35. Ji, P., S.R. Jayapal, and H.F. Lodish, *Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2.* Nature Cell Biology, 2008. **10**(3): p. 314-321.

36. Sugiyama, T., et al., *Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches.* Immunity, 2006. **25**(6): p. 977-988.

37. Allen, T.D. and T.M. Dexter, *Ultrastructural aspects of erythropoietic differentiation in long-term bone marrow culture.* Differentiation, 1982. **21**(2): p. 86-94.

38. Lee, S.H., et al., *Isolation and immunocytochemical characterization of human bone marrow stromal macrophages in hemopoietic clusters.* J Exp Med, 1988. **168**(3): p. 1193-1198.

39. An, X. and N. Mohandas, *Erythroblastic islands, terminal erythroid differentiation and reticulocyte maturation.* Int J Hematol, 2011. **93**(2): p. 139-43.

40. Korolnek, T. and I. Hamza, *Macrophages and iron trafficking at the birth and death of red cells.* Blood, 2015. **125**(19): p. 2893-7.

41. Leimberg, M.J., et al., *Macrophages function as a ferritin iron source for cultured human erythroid precursors.* J Cell Biochem, 2008. **103**(4): p. 1211-1218.

42. Chow, A., et al., *CD169 $^{+}$ macrophages provide a niche promoting erythropoiesis under homeostasis and stress.* Nat Med, 2013. **19**(4): p. 429-436.

43. Panoskaltsis, N., A. Mantalaris, and J.H. Wu, *Engineering a mimicry of bone marrow tissue ex vivo.* J Biosci Bioeng, 2005. **100**(1): p. 28-35.

44. Wang, L.D. and A.J. Wagers, *Dynamic niches in the origination and differentiation of haematopoietic stem cells.* Nat Rev Mol Cell Biol, 2011. **12**(10): p. 643-55.

45. Griffith, L.G. and M.A. Swartz, *Capturing complex 3D tissue physiology in vitro.* Nature Reviews Molecular Cell Biology, 2006. **7**(3): p. 211-224.

46. Lazar-Karsten, P., et al., *The influence of extracellular matrix proteins and mesenchymal stem cells on erythropoietic cell maturation.* Vox Sang, 2011. **101**(1): p. 65-76.

47. Li, X. and H.J. Deeg, *Murine xenogeneic models of myelodysplastic syndrome: an essential role for stroma cells.* Exp Hematol, 2014. **42**(1): p. 4-10.

48. Raaijmakers, M.H., et al., *Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia.* Nature, 2010. **464**(7290): p. 852-7.

49. Mei, Y., et al., *Age-related inflammatory bone marrow microenvironment induces ineffective erythropoiesis mimicking del(5q) MDS.* Leukemia, 2018. **32**: p. 1023-1033.

50. Palis, J., *Primitive and definitive erythropoiesis in mammals.* Frontiers in Physiology, 2014. **5**: p. 1-9.

51. Sun, S., Y. Peng, and J. Liu, *Research advances in erythrocyte regeneration sources and methods in vitro.* Cell Regeneration, 2018. **7**: p. 45-49.

52. Giarratana, M.C., et al., *Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells.* Nature Biotechnology, 2005. **23**(1): p. 69-74.

53. Neildez-Nguyen, T.M., et al., *Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo.* Nat Biotechnol., 2002. **20**(5): p. 467-472.

54. Migliaccio, G., et al., *In Vitro Mass Production of Human Erythroid Cells from the Blood of Normal Donors and of Thalassemic Patients.* Blood Cells, Molecules, and Diseases, 2002. **28**(2): p. 168-180.

55. Ma, F., et al., *Generation of functional erythrocytes from human embryonic stem cell-derived definitive hematopoiesis.* PNAS, 2008. **105**(35): p. 13087-13092.

56. Xi, J., et al., *In Vitro Large Scale Production of Human Mature Red Blood Cells from Hematopoietic Stem Cells by Coculturing with Human Fetal Liver Stromal Cells.* BioMed Research International, 2013.

57. Malik, P., et al., *An in vitro model of human red blood cell production from hematopoietic progenitor cells.* Blood, 1998. **91**(8): p. 2664-2671.

58. Christaki, E.E., et al., *Ex vivo generation of transfusable red blood cells from various stem cell sources: A concise revisit of where we are now.* Transfus Apher Sci, 2019. **58**(1): p. 108-112.

59. Giarratana, M.C., et al., *Proof of principle for transfusion of in vitro–generated red blood cells.* Blood, 2011. **118**(19): p. 5071-5079.

60. Olivier, E., C. Qiu, and E.E. Bouhassira, *Novel, high-yield red blood cell production methods from CD34-positive cells derived from human embryonic stem, yolk sac, fetal liver, cord blood, and peripheral blood.* Stem Cells Transl Med., 2012. **1**(8): p. 604-614.

61. Huang, N.J., et al., *Genetically engineered red cells expressing single domain camelid antibodies confer long-term protection against botulinum neurotoxin.* Nat Commun, 2017. **8**(1): p. 423.

62. Hu, J., et al., *Isolation and functional characterization of human erythroblasts at distinct stages: implications for understanding of normal and disordered erythropoiesis in vivo.* Blood, 2013. **121**(16): p. 3246-3253.

63. Lu, S.-J., et al., *Biologic properties and enucleation of red blood cells from human embryonic stem cells.* Blood, 2008. **112**(12): p. 4475-4484.

64. Lu, S.J., et al., *3D microcarrier system for efficient differentiation of human pluripotent stem cells into hematopoietic cells without feeders and serum.* Regen Med., 2013. **8**(4): p. 413-424.

65. Takahashi, K. and S. Yamanaka, *Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.* Cell, 2006. **126**(4): p. 663-676.

66. Lapillonne, H., et al., *Red blood cell generation from human induced pluripotent stem cells: perspectives for transfusion medicine.* Haematologica, 2010. **95**(10): p. 1651-1659.

67. Kobari, L., et al., *Human induced pluripotent stem cells can reach complete terminal maturation: in vivo and in vitro evidence in the erythropoietic differentiation model.* Haematologica, 2012. **97**(12): p. 1795-1803.

68. Fujita, A., et al., *β-Globin-Expressing Definitive Erythroid Progenitor Cells Generated from Embryonic and Induced Pluripotent Stem Cell-Derived Sacs.* Stem Cells, 2016. **34**(6): p. 1541-1552.

69. Uchida, N., et al., *Efficient Generation of β-Globin-Expressing Erythroid Cells Using Stromal Cell-Derived Induced Pluripotent Stem Cells from Patients with Sickle Cell Disease.* Stem Cells, 2017. **35**(3): p. 586-596.

70. Kurita, R., et al., *Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells.* PLoS One, 2013. **8**(3).

71. Hirose, S., et al., *Immortalization of erythroblasts by c-MYC and BCL-XL enables large-scale erythrocyte production from human pluripotent stem cells.* Stem Cell Reports, 2013. **1**(6): p. 499-508.

72. Trakarnsanga, K., et al., *An immortalized adult human erythroid line facilitates sustainable and scalable generation of functional red cells.* Nat Commun, 2017. **8**.

73. Lee, E., et al., *Defined Serum-Free Medium for Bioreactor Culture of an Immortalized Human Erythroblast Cell Line.* Biotechnol J., 2018. **13**(4).

74. Tun, T., et al., *Effect of growth factors on ex vivo bone marrow cell expansion using three-dimensional matrix support.* Artif Organs, 2002. **26**(4): p. 333-339.

75. Godbey, W.T. and A. Atala, *In vitro systems for tissue engineering.* Ann N Y Acad Sci, 2002. **961**: p. 10-26.

76. Lee, E., et al., *Red Blood Cell Generation by Three-Dimensional Aggregate Cultivation of Late Erythroblasts.* Tissue Eng Part A, 2015. **21**(3-4): p. 817-828.

77. Brafman, D.A., et al., *Long-term human pluripotent stem cell self-renewal on synthetic polymer surfaces.* Biomaterials, 2010. **31**(34): p. 9135-9144.

78. Mortera-Blanco, T., et al., *Long-term cytokine-free expansion of cord blood mononuclear cells in three-dimensional scaffolds.* Biomaterials, 2011. **32**(35): p. 9263-70.

79. Mufti, G.J., et al., *Diagnosis and classification of myelodysplastic syndrome: International Working Group on Morphology of myelodysplastic syndrome (IWGM-MDS) consensus proposals for the definition and enumeration of myeloblasts and ring sideroblasts.* Haematologica, 2008. **93**(11): p. 1712-7.

80. Nolte, F. and W.K. Hofmann, *Myelodysplastic syndromes: molecular pathogenesis and genomic changes.* Ann Hematol, 2008. **87**(10): p. 777-95.

81. Hellstrom-Lindberg, E. and M. Cazzola, *The role of JAK2 mutations in RARS and other MDS.* Hematology Am Soc Hematol Educ Program, 2008: p. 52-9.

82. Arber, A., et al., *The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia.* Blood, 2016. **127**(20): p. 2391-405.

83. Greenberg, P.L., et al., *Revised international prognostic scoring system for myelodysplastic syndromes.* Blood, 2012. **120**(12): p. 2454-65.

84. Jonas, B.A. and P.L. Greenberg, *MDS prognostic scoring systems - past, present, and future.* Best Pract Res Clin Haematol, 2015. **28**(1): p. 3-13.

85. Papaemmanuil, E., et al., *Clinical and biological implications of driver mutations in myelodysplastic syndromes.* Blood, 2013. **122**(22): p. 3616-27; quiz 3699.

86. Haferlach, T., et al., *Landscape of genetic lesions in 944 patients with myelodysplastic syndromes.* Leukemia, 2014. **28**(2): p. 241-7.

87. Karimi, M., et al., *High-throughput mutational screening adds clinically important information in myelodysplastic syndromes and secondary or therapy-related acute myeloid leukemia.* Haematologica, 2015. **100**(6): p. e223-5.

88. Papaemmanuil, E., et al., *Somatic SF3B1 mutation in myelodysplasia with ring sideroblasts.* N Engl J Med, 2011. **365**(15): p. 1384-95.

89. Yoshida, K., et al., *Frequent pathway mutations of splicing machinery in myelodysplasia.* Nature, 2011. **478**: p. 64-69.

90. Woll, P.S., et al., *Myelodysplastic syndromes are propagated by rare and distinct human cancer stem cells in vivo.* Cancer Cell, 2014. **25**(6): p. 794-808.

91. Björkman, S.E., *Chronic refractory anemia with sideroblastic bone marrow; a study of four cases.* Blood, 1956. **11**(3): p. 250-259.

92. Bennett, J.M., et al., *Proposals for the classification of the myelodysplastic syndromes.* Br J Haematol, 1982. **51**(2): p. 189-199.

93. Jaffe, E.S., et al., *Pathology and Genetics of Tumours of Haematopoietic and Lymphoid Tissues.* IARC WHO Classification of Tumours, 2001. **3**.

94. Nikpour, M., et al., *The transporter ABCB7 is a mediator of the phenotype of acquired refractory anemia with ring sideroblasts.* Leukemia, 2013. **27**(4): p. 889-96.

95. Tehranchi, R., et al., *Granulocyte colony-stimulating factor inhibits spontaneous cytochrome c release and mitochondria-dependent apoptosis of myelodysplastic syndrome hematopoietic progenitors.* Blood, 2003. **101**(3): p. 1080-6.

96. Conte, S., et al., *Aberrant splicing of genes involved in haemoglobin synthesis and impaired terminal erythroid maturation in SF3B1 mutated refractory anaemia with ring sideroblasts.* Br J Haematol, 2015. **17**(4): p. 478-490.

97. Tehranchi, R., et al., *Aberrant mitochondrial iron distribution and maturation arrest characterize early erythroid precursors in low-risk myelodysplastic syndromes.* Blood, 2005. **106**(1): p. 247-53.

98. Malcovati, L., et al., *SF3B1 mutation identifies a distinct subset of myelodysplastic syndrome with ring sideroblasts.* Blood, 2015. **126**(2): p. 233-41.

99. Malcovati, L., et al., *Clinical significance of SF3B1 mutations in myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms.* Blood, 2011. **118**(24): p. 6239-46.

100. Darman, R.B., et al., *Cancer-Associated SF3B1 Hotspot Mutations Induce Cryptic 3' Splice Site Selection through Use of a Different Branch Point.* Cell Rep, 2015. **13**(5): p. 1033-1045.

101. Boultwood, J., et al., *The role of the iron transporter ABCB7 in refractory anemia with ring sideroblasts.* PLoS One, 2008. **3**(4): p. e1970.

102. Nikpour, M., et al., *Gene expression profiling of erythroblasts from refractory anaemia with ring sideroblasts (RARS) and effects of G-CSF.* Br J Haematol, 2010. **149**(6): p. 844-54.

103. Bondu, S., et al., *A variant erythroferrone disrupts iron homeostasis in SF3B1-mutated myelodysplastic syndrome.* Sci. Transl. Med., 2019. **11**(500).

104. De La Garza, A., et al., *The splicing factor Sf3b1 regulates erythroid maturation and proliferation via TGFb signaling in zebrafish.* Blood advances, 2019. **3**(4): p. 2093-2104.

105. McKerrell, T., et al., *Leukemia-associated somatic mutations drive distinct patterns of age-related clonal hemopoiesis.* Cell Rep, 2015. **10**(8): p. 1239-1245.

106. Hsu, J., et al., *Reprogramming identifies functionally distinct stages of clonal evolution in myelodysplastic syndromes.* Blood, 2019. **134**(2): p. 186-198.

107. Claessens, Y.-E., et al., *In vitro proliferation and differentiation of erythroid progenitors from patients with myelodysplastic syndromes: evidence for Fas-dependent apoptosis.* Blood, 2002. **99**(5): p. 1594-1601.

108. Visconte, V., et al., *SF3B1 haploinsufficiency leads to formation of ring sideroblasts in myelodysplastic syndromes.* Blood, 2012. **120**(16): p. 3173-3186.

109. Visconte, V., et al., *Splicing factor 3b subunit 1 (Sf3b1) haploinsufficient mice display features of low risk Myelodysplastic syndromes with ring sideroblasts.* J Hematol Oncol., 2014. **7**(89).

110. Mupo, A., et al., *Hemopoietic-specific Sf3b1-K700E knock-in mice display the splicing defect seen in human MDS but develop anemia without ring sideroblasts.* Leukemia, 2017. **31**: p. 720-727.

111. Obeng, E.A., et al., *Physiologic Expression of Sf3b1K700E Causes Impaired Erythropoiesis, Aberrant Splicing, and Sensitivity to Therapeutic Spliceosome Modulation.* Cancer Cell, 2016. **30**: p. 404-417.

112. Cazzola, M. and L. Malcovati, *Myelodysplastic syndromes--coping with ineffective hematopoiesis.* N Engl J Med, 2005. **352**(6): p. 536-8.

113. Garelius, H.K., et al., *Erythropoiesis-stimulating agents significantly delay the onset of a regular transfusion need in nontransfused patients with lower-risk myelodysplastic syndrome.* J Intern Med., 2017. **281**(3): p. 284-299.

114. Jädersten, M., et al., *Erythropoietin and Granulocyte-Colony Stimulating Factor Treatment Associated With Improved Survival in Myelodysplastic Syndrome.* Journal of Clinical Oncology, 2008. **26**(21): p. 3607-3613.

115. de Swart, L., et al., *Impact of red blood cell transfusion dose density on progression-free survival in lower-risk myelodysplastic syndromes patients.* Haematologica, 2019.

116. Steensma, D.P. and N. Gattermann, *When is iron overload deleterious, and when and how should iron chelation therapy be administered in myelodysplastic syndromes?* Best Pract Res Clin Haematol, 2013. **26**(4): p. 431-444.

117. Ebert, B.L. and H.F. Bunn, *Regulation of the erythropoietin gene.* Blood, 1999. **94**(6): p. 1864-1877.

118. Eshghi, S., et al., *Alpha4beta1 integrin and erythropoietin mediate temporally distinct steps in erythropoiesis: integrins in red cell development.* J Cell Biol, 2007. **177**(5): p. 871-80.

119. Attie, K.M., et al., *A phase 1 study of ACE-536, a regulator of erythroid differentiation, in healthy volunteers.* Am J Hematol, 2014. **89**(7): p. 766-70.

120. Ruckle, J., et al., *Single-Dose, Randomized, Double-Blind, Placebo-Controlled Study of ACE-011 (ActRIIA-IgG1) in Postmenopausal Women*.* Journal of Bone and Mineral Research, 2009. **24**(4): p. 744-752.

121. Fenaux, P., J.J. Kiladjian, and U. Platzbecker, *Luspatercept for the treatment of anemia in myelodysplastic syndromes and primary myelofibrosis.* Blood, 2019. **133**(8): p. 790-794.

122. Platzbecker, U., et al., *Luspatercept for the treatment of anaemia in patients with lower-risk myelodysplastic syndromes (PACE-MDS): a multicentre, open-label phase 2 dose-finding study with long-term extension study.* Lancet Oncol., 2017. **18**(10): p. 1338-1347.

123. Fenaux, P., et al., *The Medalist Trial: Results of a Phase 3, Randomized, Double-Blind, Placebo-Controlled Study of Luspatercept to Treat Anemia in Patients with Very Low-, Low-, or Intermediate-Risk Myelodysplastic Syndromes (MDS) with Ring Sideroblasts (RS) Who Require Red Blood Cell (RBC) Transfusions.* Blood, 2018. **132**(1).

124. Han, Y., et al., *GDF11 is increased in patients with myelodysplastic syndrome.* Int J Clin Exp Pathol, 2016. **9**(6): p. 6031-6038.

125. Guerra, A., et al., *Lack of Gdf11 does not improve anemia or prevent the activity of RAP-536 in a mouse model of beta-thalassemia.* Blood, 2019. **134**(6): p. 568-572.

126. Iancu-Rubina, C., et al., *Stromal cell-mediated inhibition of erythropoiesis can be attenuated by Sotatercept (ACE-011), an activin receptor type II ligand trap.* Experimental Hematology, 2013. **41**(2): p. 155-166.

127. Blanco, T.M., et al., *The development of a three-dimensional scaffold for ex vivo biomimicry of human acute myeloid leukaemia.* Biomaterials, 2010. **31**(8): p. 2243-51.

128. Koenigsmann, M., et al., *Myeloid and Erythroid Progenitor Cells From Normal Bone Marrow Adhere To Collagen Type I.* Blood, 1992. **79**(3): p. 657-665.

129. Maecker, H. and J. Trotter, *Flow Cytometry Controls, Instrument Setup, and the Determination of Positivity.* Cytometry A, 2006. **69**(9): p. 1037-1042.

130. Mortera-Blanco, T., et al., *SF3B1-Initiating Mutations in MDS with Ring Sideroblasts Target Lymphomyeloid Hematopoietic Stem Cells.* Blood, 2017. **130**(7): p. 881-890.

131. Berridge, M.V. and A.S. Tan, *Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction.* Arch Biochem Biophys, 1993. **303**(2): p. 474-82.

132. Ploemacher, R.E., et al., *Use of limiting-dilution type long-term marrow cultures in frequency analysis of marrow-repopulating and spleen colony-forming hematopoietic stem cells in the mouse.* Blood, 1991. **78**(10): p. 2527-2533.

133. Ploemacher, R.E., et al., *An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse.* Blood, 1989. **74**(8): p. 2755-2763.

134. Sutherland, H.J., et al., *Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro.* Blood, 1989. **74**(5): p. 1563-1570.

135. Sutherland, H.J., et al., *Functional characterization of individual human hematopoietic stem cells cultured at limiting dilution on supportive marrow stromal layers.* Proc Nati Acad Sci U S A, 1990. **87**(9): p. 3584-3588.

136. Zandstra, P.W., et al., *Ontogeny-associated changes in the cytokine responses of primitive human haemopoietic cells.* British Journal of Haematology, 1998. **101**(4): p. 770-78.

137. Hogge, D.E., et al., *Enhanced Detection, Maintenance, and Differentiation of Primitive Human Hematopoietic Cells in Cultures Containing Murine Fibroblasts Engineered to Produce Human Steel Factor, Interleukin-3, and Granulocyte Colony-Stimulating Factor.* Blood, 1996. **88**(10): p. 3765-73.

138. Tsiatis, A.C., et al., *Comparison of Sanger sequencing, pyrosequencing, and melting curve analysis for the detection of KRAS mutations: diagnostic and clinical implications.* J Mol Diagn, 2010. **12**(4): p. 425-432.

139. Vogelstein, B. and K.W. Kinzler, *Digital PCR.* Proc Nati Acad Sci U S A, 1999. **96**(16): p. 9236-9241.

140. Hindson, B.J., et al., *High-throughput droplet digital PCR system for absolute quantitation of DNA copy number.* Anal Chem, 2011. **83**(22): p. 8604-8610.

141. Jager, W., et al., *Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays.* BMC Immunol, 2009. **10**(52): p. 1-11.

142. Mian, S.A., et al., *SF3B1 mutant MDS-initiating cells may arise fromthe haematopoietic stem cell compartment.* Nature Communications, 2015.

143. Mian, S.A., et al., *Spliceosome mutations exhibit specific associations with epigenetic modifiers and proto-oncogenes mutated in myelodysplastic syndrome.* Haematologica, 2013. **98**(7): p. 1058-1066.

144. Buckton, K.E., W.M. Brown, and P.G. Smith, *Lymphocyte survival in men treated with x-rays for ankylosing spondylitis.* Nature, 1967. **214**(5087): p. 470-473.

145. Galy, A., et al., *Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset.* Immunity, 1995. **3**(4): p. 459-473.

146. Nilsson, L., et al., *Involvement and functional impairment of the CD34(+)CD38(-)Thy-1(+) hematopoietic stem cell pool in myelodysplastic syndromes with trisomy 8.* Blood, 2002. **100**(1): p. 259-267.

147. Thanopoulou, E., et al., *Engraftment of NOD/SCID-beta2 microglobulin null mice with multilineage neoplastic cells from patients with myelodysplastic syndrome.* Blood, 2004. **103**(11): p. 4285-93.

148. Medyouf, H., et al., *Myelodysplastic cells in patients reprogram mesenchymal stromal cells to establish a transplantable stem cell niche disease unit.* Cell Stem Cell, 2014. **14**(6): p. 824-37.

149. Hino, M., et al., *Effects of type β transforming growth factors on haematopoietic progenitor cells.* Br J Haematol, 1988. **70**: p. 143-147.

150. Krystal, G., et al., *Transforming Growth Factor β1 Is an Inducer of Erythroid Differentiation.* J. Exp. Med, 1994. **180**: p. 851-860.

151. Zermati, Y., et al., *Transforming growth factor inhibits erythropoiesis by blocking proliferation and accelerating differentiation of erythroid progenitors.* Exp Hematol, 2000. **28**: p. 885-894.

152. Zhou, L., et al., *Reduced SMAD7 Leads to Overactivation of TGF-b Signaling in MDS that Can Be Reversed by a Specific Inhibitor of TGF-b Receptor I Kinase.* Cancer Res., 2011. **71**(3): p. 955-963.

153. Zhou, L., et al., *Inhibition of the TGF-β receptor I kinase promotes hematopoiesis in MDS.* Blood, 2008. **112**(8): p. 3434-3443.

154. Johnson, C.S., et al., *In vivo hematopoietic effects of recombinant interleukin-1 alpha in mice: stimulation of granulocytic, monocytic, megakaryocytic, and early erythroid progenitors, suppression of late-stage erythropoiesis, and reversal of erythroid suppression with erythropoietin.* Blood, 1989. **73**(3): p. 678-683.

155. Bessis, M., *Erythroblastic island, functional unity of bone marrow.* Rev Hematol., 1958(13): p. 8-11.

156. Migliaccio, G., A.R. Migliaccio, and J.W. Adamson, *In Vitro Differentiation of Human Granulocyte/Macrophage and Erythroid Progenitors: Comparative Analysis of the Influence of Recombinant Human Erythropoietin, G-CSF, GM-CSF, and IL-3 in Serum-.* Blood, 1988. **72**(1): p. 248-256.

157. Tsapogas, P., et al., *The Cytokine Flt3-Ligand in Normal and Malignant Hematopoiesis.* International Journal of Molecular Sciences, 2017. **18**(6): p. 1-23.

158. Wang, C.Q., K.B. Udupa, and D.A. Lipschitz, *Interferon-gamma exerts its negative regulatory effect primarily on the earliest stages of murine erythroid progenitor cell development.* Journal of cellular physiology, 1995. **162**(1): p. 134-138.

159. Belay, E., et al., *Human Cord Blood and Bone Marrow CD34+ Cells Generate Macrophages That Support Erythroid Islands.* PLOS ONE, 2017. **12**(1): p. e0171096.

160. Invernizzi, R., et al., *Vascular endothelial growth factor overexpression in myelodysplastic syndrome bone marrow cells: biological and clinical implications.* Leukemia & Lymphoma, 2017. **58**(7): p. 1711-1720.

161. Shiozawa, Y., et al., *Gene expression and risk of leukemic transformation in myelodysplasia.* Blood, 2017. **130**(24): p. 2642-2653.

162. Kornblau, S.M., et al., *Recurrent expression signatures of cytokines and chemokines are present and are independently prognostic in acute myelogenous leukemia and myelodysplasia.* Blood, 2010. **116**(20): p. 4251-4261.

163. Shi, X., et al., *The inflammatory cytokine profile of myelodysplastic syndromes A meta-analysis.* Medicine (Baltimore), 2019. **98**(22): p. e15844.

164. Zhao, Z.G., et al., *Functional characteristics of mesenchymal stem cells derived from bone marrow of patients with myelodysplastic syndromes.* Cancer Letters, 2012. **317**(2): p. 136-143.

165. McCranor, B.J., et al., *Interleukin-6 Directly Impairs the Erythroid Development of Human TF-1 Erythroleukemic Cells.* Blood Cells, Molecules, and Diseases, 2014. **52**(0): p. 126-133.

166. Verhoef, G.E., et al., *Measurement of serum cytokine levels in patients with myelodysplastic syndromes.* Leukemia, 1992. **6**(12): p. 1268-1272.

167. Giudice, V., et al., *Circulating S100A8 and S100A9 protein levels in plasma of patients with acquired aplastic anemia and myelodysplastic syndromes.* Cytokine, 2019. **113**: p. 462-465.

168. Song, Y., et al., *A highly efficient and faithful MDS patient-derived xenotransplantation model for pre-clinical studies.* Nature Communications, 2019. **10**(1): p. 366.