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An RNA-interference approach

ROMAN GALEEV
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Faculty opponent
Professor Doctor Johannes Zuber, MD, PhD
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Abstract

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Despite arguably being one of most studied adult stem cells in humans, the molecular principles governing HSC fate remain incompletely defined. In this thesis, to gain deeper insights into the mechanisms underlying renewal and differentiation, we developed a global RNAi screening paradigm targeted to human cord blood derived $CD34^+$ cells, screening for negative regulators of HSC renewal and proliferation. Using both a pre-selected and a near genome wide lentiviral library, we identified MAPK14, the Cohesin complex, and JARID2 as negative regulators of human HSCs, where attenuation of each of these genes increased HSC activity.

Additionally, we used the data from our RNAi screens as a preselection tool to identify novel regulators of malignant hematopoiesis, specifically acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS). By sequencing the preselected genes in over 400 patient samples and subsequently validating our genetic candidates in both human and murine systems, we identified NCAPD2 and SDPR as potential AML and MDS-associated genes.

In summary, in this thesis, we employed global RNAi screens targeted to primary human hematopoietic stem and progenitor cells (HSPCs). We show that this approach is a feasible strategy to identify novel modifiers of cell fate, and may also complement genome-wide sequencing approaches to guide the identification of functionally relevant disease-related genes in hematopoietic malignancies.

Key words

Hematopoietic stem cell, RNA interference, acute myeloid leukemia, myelodysplastic syndrome, genetic screens, next generation sequencing, genetic regulators

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To everyone with the tenacity to read this thesis from cover to cover ☺

"There is nothing worse than death" – Tom Riddle

"Come with me. Save yourself. You don't owe these people anymore; you've given them everything" – Selina

"Not everything…not yet" – Bruce

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ABSTRACT

Blood cell formation, also known as hematopoiesis, is maintained by a small number of undifferentiated hematopoietic stem cells (HSCs) residing in our bone marrow. These cells have the capacity to self-renew, but also give rise to all mature blood cell lineages and types, effectively generating approximately 11 million cells every second throughout the life of an individual.

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ABBREVIATIONS

AGM	aorta-gonad mesonephros
AHR	aryl hydrocarbon receptor
ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
APL	acute promyelocytic leukemia
ATAQ	assay for transposase-accessible chromatin
BMT	bone marrow transplantation
CAR	CXCL12 abundant reticular cell
CAR-T	chimeric antigen receptor
CB	cord blood
CD	cluster of differentiation
CDKN	cyclin dependent kinase inhibitor
CFC	colony forming cell
CFU	colony forming unit
CGD	chronic granulomatous disease
CGL	chronic granulocytic leukemia – effectively the same as CML
CHIP	clonal hematopoiesis of indeterminate potential
ChIP	chromatin immunoprecipitation
CLL	chronic lymphocytic/lymphoid leukemia
CLP	common lymphoid progenitor
CML	chronic myelogenous/myeloid/myelocytic leukemia
CMP	common myeloid progenitor
CRISPR	clustered regularly interspaced short palindromic repeats
DC	dendritic cell
DGCR8	DiGeorge syndrome chromosome/critical region 8
DMARD	disease-modifying antirheumatic drug

DNA	deoxyribonucleic acid
DS-AMKL	down syndrome – acute megakaryoblastic leukemia
ER	endoplasmatic reticulum
ErP	erythroid progenitor
ET	essential thrombocytosis
FACS	fluorescence activated cell sorting
FGF	fibroblast growth factor
FLT	fms like tyrosine kinase
G-CSF	granucolyte – colony stimulating factor
GM-CSF	granulocyte/macrophage – colony stimulating factor
GMLP	granulocyte/macrophage/lymphoid progenitor
GMP	granulocyte-macrophage progenitor
GvHD	graft-versus-host disease
GvL	graft-versus-leukemia
HCT	hematopoietic cell transplantation
HDAC	histone deacetylase
HIV	human immunodeficiency virus
HMA	hypomethylating agent
HSC	hematopoietic stem cell
HSPC	hematopoietic stem and progenitor cell
IFN	interferon
IL	interleukin
iPSC	induced pluripotent stem cell
JARID	jumonji, AT rich interactive domain
LSC	leukemic stem cell
LT-HSC	long-term hematopoietic stem cell
MDS	myelodysplastic syndrome
MEP	megakaryocyte-erythroid progenitor
MF	myelofibrosis

MGUS	monoclonal gammopathy of unknown significance
MHC	major histocompatibility complex
miRNA	micro RNA
MkP	megakaryocytic progenitor
MM	multiple myeloma
MPN	myeloproliferative neoplasm
MPP	multipotent progenitor
NF-κB	nuclear factor kappa B
NGS	next generation sequencing
NOD/SCID	non-obese diabetic, severe combined immunodeficiency
NSG	NOD/SCID/Il2Rγ
PCA	principal component analysis
PCR	polymerase chain reaction
PDGF(R)	platelet derived growth factor (receptor)
PRC	polycomb repressor complex
PV	polycythemia vera
RBC	red blood cell
RNA	ribonucleic acid
RNAi	RNA interference
RNP	ribonucleic protein
SCF	stem cell factor
scRNA-seq	single cell RNA sequencing
shRNA	short hairpin RNA
siRNA	small inhibitory RNA
SMC	structural maintenance of chromosomes
ST-HSC	short-term hematopoietic stem cell
STAG	stromal antigen
STK	serine threonine kinase
TAD	topology associated domain

TBI	total body irradiation
TF	transcription factor
TGF	transforming growth factor
TKI	tyrosine kinase inhibitor
TNF	tumor necrosis factor
TPO	thrombopoietin
TSG	tumor suppressor gene
VAF	variant allele frequency
VEGF(R)	vascular endothelial growth factor (receptor)
VPA	valproic acid
WAS	wiskott-aldrich syndrome
WBC	white blood cell
X-SCID	x-linked severe combined immunodeficiency

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I – Baudet, A., Karlsson, C., Safaee Talkhoncheh, M., **Galeev, R.,** Magnusson, M. and Larsson J. (2012). RNAi screen identifies MAPK14 as a druggable suppressor of human hematopoietic stem cell expansion. Blood *119*, 6255-6258.

II – **Galeev, R.,** Baudet, A., Kumar, P., Rundberg Nilsson, A., Nilsson, B., Soneji, S., Törngren, T., Borg, Å., Kvist, A. and Larsson, J. (2016). Genome-wide RNAi screen identifies Cohesin genes as modifiers of renewal and differentiation in human HSCs. Cell Reports *14*, 2988-3000.

III – Kinkel, S.A., **Galeev, R.,** Flensburg, C., Keniry, A., Breslin, K., Gilian, O., Lee, S., Liu, J., Chen, K., Gearing, L.J., Moore, D.L., Alexander, W.S., Dawson, M., Majewski, I.J., Oshlack, A., Larsson, J., and Blewitt, M.E. (2015). Jarid2 regulates hematopoietic stem cell function by acting with polycomb repressive complex 2. Blood *125*, 1890-1900.

IV – **Galeev, R.,** Yoshida, K., Karimi, M., Žemaitis, K., Lehmann, S., Hellström-Lindberg, E., Ogawa, S., and Larsson J. Identification of potential disease associated genes in acute myeloid leukemia and myelodysplastic syndrome using RNAi screens and targeted sequencing. (Manuscript)

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III – Sigurdsson, V., Takei, H., Soboleva, S., Radulovic, V., **Galeev, R.,** Siva, K., Leeb-Lundberg, L.M.F., Iida, T., Nittono, H., and Miharada, K. (2016). Bile acids protect expanding hematopoietic stem cells from unfolded protein stress in fetal liver. Cell Stem Cell *18*, 522-532.

IV – Kumar, P., Baudet, A., Beck, D., Thomas, J., **Galeev, R.,** de Jong, I., Pimanda, J., and Larsson, J. (2017). Overexpression of HMGA2 promotes the myeloerythroid biased long-term reconstitution of human hematopoietic stem and progenitor cells. *Submitted*

PREFACE

I like to say that modern medicine has developed through several distinctly defined stages, although often in canon, each relying on a unique brand of science and technology. First came the carbon-based pharmaceuticals, much due to advances in organic chemistry; drugs like painkillers, antibiotics and blood pressure medications. Then came the biomolecules, from general insulin to specific antibodies targeting specific epitopes (infliximab and alemtuzumab to name a few). And then, finally, tissue and cell therapy; transplanting cells, tissues or whole organs to cure or treat a certain condition.

This thesis addresses a tiny part of the widely used bone marrow transplantation, possibly the first of the cell-based therapies. The focus lies on the identification of genes that control the biology and fate of the cell that makes bone marrow transplantation possible; the hematopoietic stem cell.

Most of the work presented in the thesis has been done in parallel with my studies in medical school and later work at the Skåne University Hospital. It has been an amazing journey, where work in the clinic interchanged with work in the research department created a positive feedback loop of motivation, interest and energy. Meeting with and taking care of patients suffering from the diseases presented here was perhaps the greatest motivation of all.

I hope my readers will enjoy reading this thesis as much as I enjoyed writing it.

Lund, Sweden, August 2018

Roman Galeev

HEMATOPOIESIS AND THE HEMATOPOIETIC STEM CELL

1. Hematopoiesis – a brief overview

1.1. The function of the hematopoietic system

Many words in the modern languages of today's Europe stem from Greek and Latin. And so does "hematopoiesis" where "hema" means "blood" and "poies" means "to make" in Greek. The field of hematopoiesis is thus concerned with understanding how blood, with all its different components and functions, is made.

Blood is a truly unique organ devoid of solid shape, and with its liquid form, it has the ability to reach and penetrate virtually every part of the human body with a few exceptions (such as the cornea, cardiac valves and nails to name a few). There is no test that doctors do more frequently than the blood test, and the constituents of blood, likely due to the omnipresence of blood in our bodies, with all the trace elements and proteins, reflect the state in the entire organism quite remarkably.

Broadly speaking, the mature blood cells are divided into three groups; erythrocytes (red blood cells), responsible for delivering oxygen and removing carbon dioxide; thrombocytes, also known as platelets, responsible for coagulation, making sure we do not bleed out when we get an injury, and finally leukocytes (white blood cells), combating foreign micro-organisms, as well as monitoring the integrity of our own cells.

The white blood cells show the highest complexity in their mature state. There is practically one kind of erythrocytes and thrombocytes, but a wide range of leukocytes. The leukocytes are usually, in turn, divided into two major groups; the lymphoid cells, and the myeloid cells. The term "myeloid cell" is used slightly differently in the literature. My definition of myeloid cell is: "every leukocyte that is not a lymphocyte". It is tempting to say that the myeloid lineage is responsible for the innate immune defense, while the lymphoid lineage is adaptive, however, this would be an oversimplification.

Myeloid cells are usually described as the "first-line" of defense. They are not specific for a particular virus, bacteria, fungus, or protozoa, and respond fairly similarly to all kinds of infections. Myeloid cells consist of granulocytes, monocytes/macrophages and mast cells.

There are three types of granulocytes; neutrophils, eosinophils and basophils. Neutrophils, the most abundant granulocyte in our body, attack primarily bacteria using phagocytosis, a process of taking up the foreign invader into its own cytoplasm for digestion and degranulation; release of toxic substances extracellularly, combating bacteria in the tissues (Witko-Sarsat et al., 2000). Eosinophils are the main arsenal against parasitic infections. Naturally, parasites are usually too big to be ingested, and the attack against them is primarily an extracellular one using degranulation of toxic substances stored inside the eosinophils. Eosinophils also have a role in combating viral infections, and together with the mast cells, play a critical role in development and maintenance of allergies and asthma (Uhm et al., 2012). The role of basophils, the rarest granulocyte, and also the one least studied appears to be fairly similar to eosinophils. They also fight parasites, ectoparasites (parasites that live outside the body like lice, fleas and mites) being their main preference and like the eosinophils, contribute to the pathogenesis of allergies and asthma (Mukai and Galli, 2013).

Monocytes themselves have little function. They circulate the blood waiting for cues from granulocytes and the adaptive immune response to migrate into tissues. There, they transform into macrophages that phagocytose foreign substances and invaders. Contrasting with granulocytes, macrophages do not secrete granules and their main function is strictly phagocytosis. Cells of the adaptive immunity, mainly T and NK cells, stimulate and activate the macrophage (Nichols et al., 1971; Swirski et al., 2009).

The cell types I have mentioned above have, in turn, several sub-groups. One worth mentioning is the osteoclast, a macrophage exclusive to bone that remodels and strengthens the bones together with the osteoblast. Both the osteoblast and the osteoclast have a role in the regulation of HSC function (Vaananen et al., 2000).

Finally, the role of the mast cell is not as clear-cut. It is primarily known as the main culprit behind allergies and asthma, diseases that continue to increase more and more in Western countries. However, they also have a role in immune tolerance, pathogen defense and blood-brain barrier function (da Silva et al., 2014; Polyzoidis et al., 2015).

A critical role of myeloid cells not discussed as often is wound healing and regenerative medicine in general, making them critical players in scar formation, regeneration and aging. After hemostasis is achieved by platelets, myeloid cells play a critical role in keeping the invading bacteria out as well as clearing out all the damaged and dead tissue before development of new blood vessels (angiogenesis) and collagen deposition occurs to rebuild the connective tissue. Lastly, re-epithelialization repairs the broken skin and the wound closes (Stadelmann et al., 1998).

Lymphoid cells, in turn, are made of three distinct sub-lineages; the B-cells ("B" standing for "bursa of Fabricius" an unique organ in birds where B-cells were firstly discovered) (Stadelmann et al., 1998), T-cells (T standing for thymus) and the natural killer (NK) cells. B-cells produce antibodies that recognize specific molecules or epitopes on foreign invaders and help the innate immune system to destroy them (Stadelmann et al., 1998). T-cells work as master regulators of the immune response in general, activating other cells to produce antibodies or phagocytize pathogens (Buchholz et al., 2016). NK-cells work primarily on their own, and are more focused on problems within our own bodies, such as killing off virus-infected cells or cells that have transformed to cancer.

Finally, the source of dendritic cells (DC) is not as clear-cut as for the rest of the leukocytes. Their origins have been traced back to progenitors of both the myeloid and the lymphoid lineages. DCs are primarily residing in tissues close to the external environment; such as the mucosa of the gastrointestinal tract or the epidermis of the skin. They digest foreign substances and present them to T-cells residing in a lymph node nearby, thus primarily functioning as first informants of a coming microbial invasion (Vivier et al., 2011).

Blood cells have one of the highest turnover rates in the body. There have been huge variations in the proposed number of cells in an adult human being from different sources and different methodologies. In an attempt to pool the existing data together, it has recently been estimated and proposed that the human body contains approximately $3.72 \cdot 10^{13}$ cells where as much as 70% is blood cells and 94% of the blood cells, in turn, are erythrocytes. (Bianconi et al., 2013). Different blood cells have vastly different lifespans, with neutrophils lasting 1-5 days in contrast to memory T-cells that live almost as long as the organism.

To keep up with this demand, the body needs to make approximately 10^{12} blood cells every day which is the equivalent of approximately 11 million cells every second (Ogawa, 1993). To ensure and enable such a tremendous output, HSCs go through differentiation into stage-specific progenitors undergoing proliferative expansion, increasing the cell number and restricting stem cell plasticity at each stage until the final, mature effector cell stage, is reached (Figure 1A). Or at least, this has been the prevailing paradigm of hematopoiesis for several decades. Lately however, the integrity of defined progenitor populations in particular have come into question. The alternative model argues for a more continuous differentiation process, where cells do not go through specific stages, but rather move through a continuum, from HSCs to mature effector cells (Figure 1C). This topic will be covered in greater detail in part 2.3.

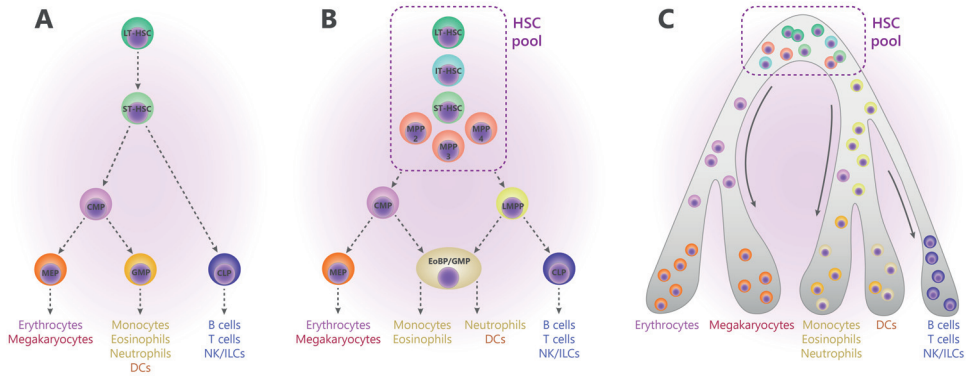

Illustration: Veronika Bendoriūtė

Figure 1. Evolution of the model of the hematopoietic hierarchical tree. **A,** the first model; a homogeneous pool of LT-HSCs goes through several distinct intermediate progenitor stages before terminally differentiating into mature effector cells. **B,** the revised model, initially described in 2007; the HSC pool contains several different types of HSCs, each with a certain lineage preference. The myeloid/lymphoid branching points does not occur as early as previously thought; cells retain their lineage plasticity further down in the tree. **C,** The revised model of the hematopoietic hierarchy in 2018. HSCs are unique and lineage restricted, and their path of differentiation is, to some extent, predefined. There are no clear intermediate differentiation stages, rather, cells move along a continuum, from LT-HSC to mature cells.

1.2. Hematopoiesis throughout life – from the fetus to the supercentenarian

Due to ethical constraints, studying developing hematopoiesis and the way blood formation changes during life is particularly difficult in humans. The vast information we have on this topic, particularly the details, comes from studies done in mice.

In the very early stages of gestation, where differentiation just reached the morula and blastocyst stages, nutrient supply and waste removal is achieved through diffusion. However, quite soon after implantation this is not enough and some form of circulation is required (Palis, 2016).

Interestingly, the human hematopoietic system develops in "waves"; transient proliferation of hematopoietic cells originating in different sites of the developing embryo, without any clear-cut "stem" cells. Intriguingly, this is, in effect, hematopoiesis without HSCs. The site of the first wave is in the human yolk sac, where hematopoietic cells can be detected as early as day 15 (Tavian and Peault, 2005). The yolk sac forms cells almost exclusively of the erythroid lineage, with only few monocyte/macrophages and megakaryocytes and no lymphoid cells. The yolk sac remains the primary hematopoietic organ of the embryo until the 6th week (i.e. the 8th week of pregnancy), when this role is taken over by the fetal liver (Palis and Yoder, 2001).

The fetal liver is seeded with hematopoietic progenitor cells from the yolk sac around day 23 (Gomez Perdiguero et al., 2015; McGrath et al., 2015), but there is also a second wave of hematopoiesis that originates from the wall of the newly formed aorta around day 27 and seeds the liver around day 30 where the HSCs expand at least two orders of magnitude (Ivanovs et al., 2011). There are several controversies regarding the process that follows, particularly the origin of the multilineage, long term reconstituting HSCs. While some authors claim that the yolk sac, at least partially contributes to LT-HSCs where the primitive HPCs mature into true LT-HSCs and eventually expand in the fetal liver before colonizing the bone marrow to sustain lifelong hematopoiesis (Lee et al., 2016; Samokhvalov et al., 2007), others claim that definitive LT-HSCs are produced *de novo* from the ventral wall of the dorsal aorta (Medvinsky and Dzierzak, 1996; Muller et al., 1994).

Regardless, it is likely that the primitive HPCs fulfill the oxygen needs of the rapidly growing embryo, while the cells emerging from the second hematopoietic wave are capable of not only long-term engraftment but also exhibit all-lineage potential, making them complete LT-HSCs.

Since the HSCs disappear from the aorta around day 40, the fetal liver maintains the intraembryonic hematopoiesis until the gradual overtake (beginning in 10^{th} week of gestation, i.e. the 12^{th} week of pregnancy) of hematopoiesis by the bone marrow which eventually becomes the primary hematopoietic organ throughout life (Cheng et al., 2000; Christensen et al., 2004). Out of all HSCs studied throughout development and life, the ones emerging from the aorta display the highest activity, likely due to their requirement of expansion as they migrate into the fetal liver (Bianconi et al., 2013).

While it is difficult to study embryonic and fetal hematopoiesis in humans, neonatal sources, i.e. cord blood, is likely the most accessible source of human HSCs today. After a child is born, the umbilical cord is cut. After 15 to 30 minutes, the placenta is detached from the uterus and is vaginally expelled as well. During gestation, the placenta has many functions, one of which is to act as a membrane or a barrier, separating the systemic circulation of the mother from her child, allowing only certain molecules and substances to pass. The placenta is therefore filled with both maternal and fetal blood when expelled, but the intraplacental membrane separates the blood. By applying pressure on the placenta, neonatal blood can easily be harvested (squeezed out) through the umbilical cord; an outstanding HSC source that before this discovery was completely disregarded and discarded (Broxmeyer et al., 1989).

Through development and aging, the number of phenotypic HSC, defined as having a certain combination of cell surface receptors, increases while their activity decreases. HSCs with the highest activity and potential for self-renewal

are found in the wall of the aorta in the embryo, with gradual decline throughout the developmental stages; fetal liver, cord blood, young bone marrow and finally old bone marrow (Holyoake et al., 1999; Lansdorp et al., 1993; Pawliuk et al., 1996; Rebel et al., 1996a; Rebel et al., 1996b; Rundberg Nilsson et al., 2016; Weekx et al., 1998).

It is an intriguing question, why the number of HSCs (as determined by their cell surface marker expression) increases while the actual HSC activity decreases. The deeper biological meaning behind this change is unclear, whether there are signaling cues that push cells into self-renewal vs. differentiation, or if it is a compensatory mechanism in body homeostasis due to decreased output of mature effector cells.

In general, hematopoiesis of older people displays slight abnormalities in their blood cell profiles, the most common one is anemia that is not due to inflammation, infection or deficiency of iron, folate, or cobalamins. There is also a "myeloid skewing" in the old hematopoietic system, where relatively more myeloid than lymphoid cells are produced. The overall myeloid output is lower in the old individuals; the lymphoid output simply decreases even further. Often, the myeloid skewing is coupled with the development and establishment of clonal hematopoiesis. In newborns and young adults, thousands of different HSCs give rise to mature effector cells. In older people, this diversity diminishes and fewer and fewer pools of seemingly identical stem cells form the output. In a whole-genome sequencing effort of a 115-year-old healthy woman, it was discovered that she only had two clones of HSCs maintaining the whole hematopoiesis (Holstege et al., 2014). This case study was particularly interesting in regards to the other mutations found in her blood. It was discovered that her blood cells harbored near 450 mutations, but luckily none of them were cancerous. Random mutations in HSCs are known to be accumulated with time and this of course raises the question whether it therefore is only a matter of time before a random mutation strikes a critical gene, leading to development of leukemia.

Overall, the developing and aging hematopoiesis can be summarized as emerging from the aorta with a high stem cell activity, expanding in the fetal liver before seeding the bone marrow. Throughout life, stem cell activity progressively decreases, as does the overall hematopoietic output coupled with myeloid skewing and clonal hematopoiesis, often resulting in anemia, increased risk of malignant transformation and in the end, mortality and death.

2. Discovery, isolation and biology of the hematopoietic stem cell

2.1. Discovery

Due to advances in microscopy, the German pathologist Franz Neumann concluded, in the late 19th century, that blood formation had to be in the bone marrow. The Russian scientist Alexander Maximow developed this theory to include a common cell, located somewhere in the bone marrow that had to give rise to the blood cells Neumann saw (Maximow, 1909). Doubtlessly, this theory was met with extreme skepticism at the time, but it was here, with Maximow, and with blood, that the concept of hematopoietic stem cell research, and stem cell research in general, began.

There were many studies indicating the presence of a stem cell or at least a precursor cell following the nuclear bombings in 1945 (the cause of death of the lowest lethal irradiation dose was bone marrow failure), where lethally irradiated mice were saved by injection of cells from the bone marrow or spleen harvested from another mouse, but they were not aiming at the stem cell concept, rather it was the restoration of the blood, as a whole, that was in focus here.

In 1961, a wider range of effects of a bone marrow transplantation following lethal irradiation were studied by Ernest A. McCulloch and James E. Till. They discovered that injected bone marrow cells gave rise to colonies in the spleen of transplanted animals, and that the amount of colonies formed was directly proportional to the amount of bone marrow cells injected (approximately 1 colony for every 7000 cells injected) (Till, 1961). The colonies formed, denoted CFU-S (colony forming unit-spleen), gave rise to multilineage (except lymphoid; these colonies are difficult to achieve *in vitro* and *in vivo*) colonies, clearly demonstrating the multilineage potential of bone marrow cells. To assay that the colonies arose from a single cell, chromosomal breaks were introduced by sub-lethal irradiation. In the subsequent analysis it was shown that each colony had its own set of chromosomal abberations and that every cell in that colony had the same karyotypic change (Becker, 1963).

As it later turned out, cells giving rise to colonies in the spleen of lethally irradiated recipient mice were actually progenitors and not HSCs. HSCs form colonies quite rarely and when they do, it usually occurs at a later time point (after 12 to 14 days), as compared to progenitor derived colonies that can be visualized as early as after 8 days (Morrison and Weissman, 1994; Na Nakorn et al., 2002).

Cells residing in our bone marrow, while looking quite similar under the microscope, especially as far as the HSCs and restricted progenitors are concerned, are in fact quite heterogeneous and it was not until the development of fluorescence activated cell sorting (FACS) that the actual stem cells could be isolated.

2.2. Isolation

Isolation of murine HSCs predated the isolation of human HSCs. Starting in the 80s, many cell surface markers have been identified that enrich for HSC activity in the mouse. However, no combination has been able to isolate absolutely pure HSCs. An intuitive finding is the absence of lineage specific markers, i.e. HSCs do not express proteins associated with a distinct differentiated lineage, referred to as "Lin" (Spangrude et al., 1988; Uchida and Weissman, 1992). Additional markers commonly used today are c-kit (CD117) (Ikuta and Weissman, 1992), stem cell antigen 1 (sca-1) (Spangrude et al., 1988; Uchida and Weissman, 1992), CD48 (Kiel et al., 2005), CD150 (Kiel et al., 2005), CD34 (Osawa et al., 1996), and Flt3 also known as Flk2 (Christensen and Weissman, 2001). The combination $Lin^{-}Sca1^{+}c\text{-}kit^{+}$ is usually referred to as LSK. To obtain a sufficient number of LT-HSCs, all combinations are rarely used together, and the majority of studies today use either the $LSKCD34^{-}flk2^{-}$ or the $LSKCD48^{-}CD150^{+}$ combination to obtain a reasonable number of LT-HSCs with an adequate purity (approx. 1 in 3-4).

If a higher degree of purity is required, the ability of LT-HSCs to expel substances from their cytosol through membrane-bound pumps can be used. In the murine system, it is possible to isolate HSCs to 96% purity (i.e. in that given population, 24 out of 25 cells are pure, engraftable HSCs) (Matsuzaki et al., 2004). For most studies though, the sacrifice of yield for purity is to great.

Finding markers that isolate human HSCs with the same precision has been difficult. CD34 was the earliest marker for human HSCs discovered and is still of great use today (Civin et al., 1984). It took several years before additional markers that further define the human HSPC population were discovered. Perhaps it was due to difficulties in obtaining human material, or the primary research interest was more focused on the murine system. Possibly, studying the murine hematopoiesis was considerably easier and/or there was no real clinical demand; patients receiving BMT were given whole bone marrow transplants, not particular isolated cellular subsets. Eventually, additional markers for human HSPCs were discovered. CD45RA emerged as a novel marker in 1990, marking more mature cells, where it was shown that cell populations enriched from HSCs are $CD45RA^{-}$ (Lansdorp et al., 1990). CD90 (Baum et al., 1992) and CD38 (Larochelle et al., 1996) were discovered soon after. A combination of these markers later led to

subfractionation of human HSPCs into HSCs, MPP and more commited progenitors (Majeti et al., 2007). CD133 was proposed to mark engrafting human hematopoietic HSPCs (Hess et al., 2006), but this marker did offer any additional purification, particularly if CD90 was included.

The most recently discovered human HSPC marker is CD49f (Notta et al., 2011). But even with the most stringent marker combination of $CD34^{+}38^{-}90^{+}45RA^{-}49f^{+}$ and including the efflux of dye by the membrane pump as for murine HSCs, the purity of HSCs barely reaches 10%. The Endothelial Protein C Receptor (EPCR) has recently been shown to be a promising candidate for human HSPC isolation, but this study failed to take $CD49f^{+}$ into account and more research will have to be done to ascertain whether EPCR truly can narrow down the human HSC population further (Fares et al., 2017).

Interestingly, the marker combination for different populations through ontogeny (human fetal liver, cord blood, young and old bone marrow) has proven to be remarkably consistent.

2.3. Biology of HSPCs

2.3.1. HSCs

Studying human HSCs is considerably harder than their murine counterpart. Measuring the division rate for instance, or even something as simple as total number of HSCs in the human body in steady state is not an entirely trivial task. Using cell labeling studies to study human HSCs *in vivo* is not only technically challenging, but also ethically completely indefensible. We are therefore left to do such measurements indirectly, and through simulation and modeling. Using hemizygous selection (i.e. the fact that different HSCs in human females have alternate imprinting of the X-chromosome, and measuring the changes in this ratio) one study estimated the number of HSCs in an adult human to be approximately only 3 000 – 10 000 cells (Catlin et al., 2011), and these cells have to produce approximately 10^{11}-10^{12} every day or around 100 million cells every second throughout the lifetime of a human being. For a long time, the hematopoietic system was viewed as a rigid hierarchical pyramid, or tree as depicted in Figure 1A. At the top were the LT-HSCs; the only true stem cells, a relatively homogeneous population displaying multipotency, self-renewal and long-term engraftment/blood cell production capabilities (Orkin and Zon, 2008). The way so few cells achieved this tremendous output was believed to be due to a vast number of progenitor cell stages, where cells originating from the asymmetric division of the multipotent HSCs go through different progenitor cell stages as depicted in figure 1A, with exponential amplification of cell number and subsequent lineage restriction. In this model, long-term self-renewal is lost first;

ST-HSCs and MPPs are capable of self-renewal but just within a limited timeframe while remaining multipotent. Complete multipotency is lost at the first branching point, where cells become either myeloid, erythroid and megakaryocytic or lymphoid precursors. These two progenitor populations; CMP and CLP, differentiate further to more lineage specific precursors, becoming bi- and eventually unipotent, while at every stage greatly increasing the cell number (Figure 1A).

During the last decade, this model has become increasingly challenged, and today, it is safe to say that it is a gross oversimplification. It is primarily the ability to increase cell resolution, obtaining quality data from fewer cells and thus decreasing the noise present in bulk analysis, that demanded a revision of the hematopoietic tree. The LT-HSC compartment appeared not to be as homogenous as previously thought; rather, cells within this population display different degrees of skewing and differentiation preference; using single cell transplantation, it was shown that LT-HSCs exhibit one of four distinct differentiation patterns (Dykstra et al., 2007). Further, data suggested that the MEP population, while still believed to mainly arise through maturation and differentiation of the MPP and CMP, could develop from ST/LT-HSCs directly, bypassing the hierarchical pyramid (Adolfsson et al., 2005; Notta et al., 2016; Yamamoto et al., 2013). Additionally, the clear separation between the lymphoid and other lineages became blurred, or at least pushed significantly further down in the hierarchy (Figure 1B) (Gorgens et al., 2013).

During the last years, genetic and fluorescent labeling emerged as a pivotal strategy to study murine HSC function. In this method, a unique barcode is introduced into the genome of a cell after exposure to a stimulus. Since this barcode distributes fairly randomly, each cell will have a unique integration site. Analyzing the integration sites at different time points and different cells will allow the identification of the cell of origin for different mature cells as well as progenitor cells. In 2014, a paper questioned the contribution of LT-HSCs to mature effector cells altogether (Sun et al., 2014). This study showed that LT-HSCs had very little barcode overlap with mature cells, while the different MPP populations did, arguing that at steady-state, MPPs appear to be the primary source of mature blood cells. Following on the ST-HSC to MEP shortcut, a follow up study by the same group in 2018, questioned the existence of the MEP cell in steady-state altogether, and further showed that it is LT-HSCs, rather than ST-HSCs that give rise to platelets directly, bypassing several other progenitor cell stages (Rodriguez-Fraticelli et al., 2018). While these findings have a huge potential to cause a paradigm shift in the field of hematopoiesis, they should be interpreted with caution, particularly since the transposase labeling requires cell division which likely results in a fairly small amount (and fraction) of HSCs being

labeled. Further, results are likely to differ when conducting single cell vs. bulk assays, and also when studying hematopoiesis in steady-state vs. transplantation.

Studies using fluorescent protein tagging of HSCs in the cytoplasm or nucleus (where cell division is not as critical of a requirement) has shown that HSCs contribute to a substantially higher degree to multilineage hematopoiesis than what was deduced after analyzing data acquired using the transposon barcode approach (Busch et al., 2015; Sawai et al., 2016; Sawen et al., 2016). Nonetheless, while these studies provided compelling proof of reestablishing LT-HSCs at the top of the hierarchy of blood cell production during steady state, they indicated strongly that single LT-HSCs were doing so in a fairly lineage restricted manner. This started to bring doubt on the established 4 sub-type model of LT-HSCs that was done in bulk.

Two studies published this year have brought additional light on this topic (Carrelha et al., 2018; Rodriguez-Fraticelli et al., 2018). While the methodology in these two studies was quite different (steady-state and barcode labeling vs. single cell transplantation), several common conclusions can be drawn. LT-HSCs appear to be uni-, bi-, tri- or multipotent. The unipotency is observed only for the platelet lineage, with subsequent add-on of erythrocytes, myeloid cells, B-cells and finally T-cells. Interestingly, this is not observed *in vitro*, where the same LT-HSCs show complete multilineage potential including generating T-cells. The lineage restriction was propagated upon transplantation into secondary recipients but alleviated when cells were put in culture. While this subgrouping is tentative and needs to be further validated and elucidated, there is a general consistency using different experimental approaches in addressing the same question. Additionally, the long-term engraftment kinetics of the lymphoid lineages seem to be decoupled from the other lineages; cells giving rise to B and T-cells long-term show only transient engraftment of the other lineages (Carrelha et al., 2018).

While it is now fairly well established that lineage priming occurs early, and there appears to be significantly less plasticity at single cell level, at least *in vivo*, than was previously described, it is quite unclear how the lineage commitment is achieved. Using Mx1-Cre-induced fluorescent tagging of HSCs, Yu et al. did not observe any distinct transcriptional differences between LT-HSCs with distinct differentiation patterns, which raises the question at what level this is controlled (Yu et al., 2016). Likewise, Rodriguez-Fraticelli et al. found that while LT-HSCs are subdivided into several different groups based on principal component analysis (PCA) of single cell RNA-sequencing (scRNA-seq), this data provides little information about the differentiation pathways these cells will take, apart from the platelet lineage, which is the only mature lineage observed at the LT-HSC level (Rodriguez-Fraticelli et al., 2018). Instead, the different MPP populations (MPP1-4) were genetically primed for further differentiation, with skewing patterns

different from the 4 LT-HSC subsets identified over 10 years earlier. However, while the different MPP populations give rise to cells of different lineages with certain skewing ratios at bulk level, single-cell MPP also appear to be committed to a certain specific mature effector cell.

It seems that lineage priming of LT-HSCs appears to be established at the epigenetic level, as there are profound differences in histone methylation and accessible chromatin around enhancers and promoters of lineage specific genes (Ugarte et al., 2015; Yu et al., 2016). Possibly it is the epigenetic marks that determine which differentiation path a certain LT-HSC will take. A confusing observation is though how it is possible that no transcriptional differences are observed despite certain genes being epigenetically activated, consistent with the idea that transcriptionally, LT-HSCs appear to be fairly homogeneous. If a certain lineage gene has open enhancers and active promoters, why are they then not transcribed at a higher level than genes that are epigenetically "closed"? There could be several explanations for this. The most obvious one is the requirement of transcription factors to bind the open enhancers and promoters that may not be present at the undifferentiated stage. The genes could also be post-transcriptionally silenced. Taken together, this implies that, at least *in vivo*, there is much less plasticity than previously imaged which likely is compensated for by increased heterogeneity.

2.3.2. Progenitors

At the progenitor cell level, it was previously believed that the plastic state was established and maintained by (amongst others) expression of transcription factors of different lineages. As an example, GMPs were thought to express both CEBPα (required for neutrophil differentiation) as well as PU.1 (required for monocyte differentiation) (Scott et al., 1994; Suh et al., 2006). And at the bulk level, this was indeed the case. However, when analyzing single cells, cells that expressed multiple lineage-specific transcription factors were not found (Hoppe et al., 2016). Rather, just as LT-HSC, cells within the phenotypic GMP-compartment were heterogeneous and committed to a certain distinct mature effector cell. Also, self-renewal turned out to be not as exclusive to HSCs as was previously thought. GMP are capable of activating a self-renewal program in emergency myelopoiesis (Herault et al., 2017). It is interesting to speculate whether it is this very same self-renewal program that gets "hijacked" in acute myeloid leukemia (AML), since fusion-protein leukemias are believed to arise at this level, and normal karyotype leukemia, while initiated in HSCs, also likely become established at the "GMP-stage" (Krivtsov et al., 2006).

Taken together, these studies indicate that lineage specification and commitment occur much earlier than previously thought, likely even at the LT-HSC level. This clearly illustrates the power of the resolution achieved at the single-cell level.

These studies challenge the notion that distinct progenitor populations such as CLP, CMP and GMP even exist. Rodriguez-Fraticelli et al. failed to identify MEPs in steady-state hematopoiesis even at bulk level (Rodriguez-Fraticelli et al., 2018). While there appeared to be phenotypic, functional and molecular characteristics of HSPCs at the bulk level that enabled us to group them into the above-mentioned groups, in reality, single cells might just as well be along a continuous, and not discreet as previously imagined, differentiation spectrum from LT-HSCs to mature effector cells of the blood (Figure 1C). Furthermore, the plasticity of HSPC populations at bulk level (multi, tri, bi), appears to be severely attenuated when examining the hematopoietic system at the single-cell level.

Naturally, if stem cells are primed and show little plasticity from the very beginning, this raises the question how the hematopoietic system is then able to quickly respond to specific demands in production of certain cells. The priming of LT-HSCs towards the two most abundant lineages (platelets and erythrocytes) is certainly one factor, the ability of more downstream progenitors (such as GMPs) to self-renew is another.

While these studies answer many pivotal questions regarding HSC biology, they likely ask even more. It is unclear how well this translates to human cells and which clinical relevance this has at this time. While single cell transplantations can be done for human HSCs, tracking human hematopoiesis in steady-state will be much more challenging if possible at all. The ethical question of using such a vast number of mice as is necessary for robust single-cell transplantations should also be kept in mind. Additionally, despite the remarkable similarities between murine and human hematopoiesis, it is hard to know whether these findings are translatable to humans at all since the mice share identical genetic background, are kept in very clean environments with no exogenous stressors and are required to sustain hematopoiesis for much shorter time given their lifespan. Exogenous stressors are particularly relevant for human HSC biology where a quite diverse range of different stressors; inflammation (Takizawa et al., 2012), acute and chronic infections (Hirche et al., 2017; Matatall et al., 2016), DNA damage (Milyavsky et al., 2010; Mohrin et al., 2010), metabolic stress and autophagy (Warr et al., 2013), ER stress (Sigurdsson et al., 2016), replication stress (Flach et al., 2014), and even less obvious factors such as psychosocial stress (Heidt et al., 2014) and obesity (Ambrosi et al., 2017) affect HSC-function.

3. Studying human HSCs and hematopoiesis

3.1. Acquisition of human HSCs

Studying human HSCs is quite difficult compared to murine. There is just as much scientific effort devoted to understanding how human HSCs are regulated and function, as to developing new technologies, assays and enrichment and isolation strategies for those same purposes. The nature of this difficulty is three-fold; first, true HSCs are a very rare population in the bone marrow, Second, even with an abundant source, it is hard to isolate "homogenous" HSCs, because they are not particularly homogenous; in the human system where we can only enrich for 10% HSCs using the most stringent cell surface marker combination, the other 90% work as quite substantial confounding factors (Notta et al., 2011). Thirdly, HSCs lose their stem cell activity quite rapidly when removed from their natural environment. There is therefore only a short window of opportunity for characterizing these cells once they are isolated for analysis.

Adult human HSCs are usually derived from healthy volunteers through bone marrow aspirations. Acquisition of HSCs from old individuals is not as straightforward; it is not ideal to subject older people to unnecessary invasive procedures. Therefore, the source is usually limited to the femoral head extracted during hip replacement surgery. These patients often have dislocated fractures in the neck of the femur which cuts of the blood supply to the femoral head for a significant period of time in an anatomical location that has a remarkably poor blood supply to begin with. The lack of blood supply to HSCs later isolated from this region has caused controversy regarding to which conclusions can be drawn about old human HSC biology using this source.

As an alternative to adult bone marrow derived HSCs, umbilical cord blood is often used instead, due to its non-invasive acquisition procedure. And while there are many similarities between human HSCs from different ontogenic sources, there are also notably differences, particularly in stem cell activity, and results acquired using one source may not always necessarily be transferable to another.

3.2. Analysis and characterization of human HSCs

With the hallmark experiments of Till and McCullough (Till, 1961), the colony forming unit (CFU) was established, and is still widely used to this day. In this assay, undifferentiated cells are placed into a semi-solid medium containing cytokines that enable and promote differentiation. After two weeks, the formed colonies are scored on type (i.e. which mature cells were the plated cells able to

produce), as well as size of the colony (measuring the proliferative potential). An expansion on this technique is the long-term culture-initiating cell (LTC-IC) assay. Here, only one cell per well is plated and their ability to grow long term in culture is evaluated. As it later turned out, both these assays were in fact assaying the potential of progenitor populations and not true HSCs, and we have to this day no *in vitro* assay to assess the function of the HSC. The increased demand brought on cells through serial replating could be an option and we have seen that human HSCs are able to form colonies more times than progenitors (Galeev, R. and Larsson, J. unpublished data). The assay is likely still too unreliable though, as the ability to form colonies several times might as well be an effect of increased progenitor, and not stem cell, activity.

Another commonly used *in vitro* assay is the ordinary long-term culture, assaying the effect of various conditions on HSPC activity and differentiation. Sometimes, co-culture with stromal cells is used. Depending on the identity of the stromal cells, HSPCs can maintain their activity longer and/or enable differentiation towards the lymphoid, and particularly T-cell, lineages.

To evaluate true HSC function, *in vivo* experiments are required. The gold standard assay for HSC function is transplantation; a lethally irradiated recipient mouse is transplanted with donor HSPCs, usually with stromal support and or/competitor cells, where the observed levels of chimerism provide a direct measurement of HSC function. Since the strains are congenic there is no immune system response and the existence of two isoforms of the marker CD45 (CD45.1 and CD45.2, expressed on the majority of hematopoietic cells) makes it possible to track dynamics of donor vs. host HSC activity. However, as CD45 is not expressed on mature erythrocytes or platelets, even the *in vivo* assays carry a risk of severe result skewing given that only leukocytes and upstream progenitors are analyzed.

When assaying human HSCs, the immunological mismatches need to be considered. If human HSCs were to be transplanted into an immunocompetent mouse, the mouse immune system would mount a response and destroy the foreign cells. For this reason, human HSCs can only be assayed in immunodeficient mice. Initially, $Rag^{-/-}$ mice were used; Rag (Recombination-activating gene) is required for generating the diverse repertoire of B and T cells, and deleting this gene effectively reduces the recombination at least 1000-fold, severely crippling the ability of these mice to mount an immune response to human cells (Mombaerts et al., 1992; Oettinger et al., 1990). To improve upon the human engraftment levels seen in $Rag^{-/-}$ mice, the NOD/SCID mouse was developed in the middle to late 1990s. NOD (Non-obese diabetec), or rather NOD/ShiLtJ, reduces innate immunity through defects in macrophage activity, reduced dendritic cell function and absence of the hemolytic complement system,

while SCID attenuates the levels of B and T cells even further. Interestingly, the NOD mouse was shown to harbor a polymorphism in the Sirpa gene (expressed on mouse macrophages and found to bind to human CD47; the "don't phagocytose me" signal), decreasing the monocyte/macrophage response against the transplanted human cells (Greiner et al., 1998; Shultz et al., 2005; Shultz et al., 1995; Takenaka et al., 2007).

The most commonly used mouse model today is a slight improvement of the NOD/SCID; called NSG ("NS" standing for NOD/SCID). This is a NOD/SCID mouse where there is an additional deletion in the interleukin 2 receptor gamma chain (IL2Rγ), a subunit that is common for several other interleukin receptors (Cao et al., 1995). This effectively removes all lymphoid cells, including NK, thereby, in theory at least, overcoming the last major obstacle in the generation of an efficient xenograft transplantation model (Shultz et al., 2005). While this strain remains the gold standard to evaluate and model human hematopoiesis today, there are still several shortcomings.

First, the NSG model, while enabling higher levels of engraftment that the older NOD/SCID or the $Rag^{-/-}$ mice allows the analysis of virtually only one mature hematopoietic lineage; the leukocytes. Evaluating erythropoiesis and thrombopoiesis is considerably harder for the same reasons as described above for the murine system. Second, the engraftment is severely skewed towards the lymphoid lineage of the leukocytes and B-cells in particular. While the majority of leukocytes produced in steady state in the adult human being are myeloid, the myeloid chimerism reaches only an average of 10% in the NSG mouse. Third, engrafting leukemic cells has surprisingly proven to be quite difficult, where barely 50% of primary leukemic samples engraft. This is seemingly counterintuitive since leukemia, and AML in particular, can quickly take over the human bone marrow. A possible explanation (discussed in greater detail in part 3 of this thesis) could be niche factors; leukemias tend to remodel the niche to support the development and maintenance of malignant hematopoiesis through altered cytokine profile and signal interpretation. In the NSG mouse, the xenobiotic and "healthy" nature of the murine bone marrow could prove inhospitable to primary leukemic cells (Schepers et al., 2015).

To allow more efficient myeloid engraftment, and leukemia in particular, NSG mice have been engineered to express three human cytokines, all shown to play important roles in development of myeloid malignancies, in the murine bone marrow; stem cell factor (SCF), granulocyte/macrophage–colony stimulating factor (GM-CSF) and interleukin 3 (IL3). These mice, termed NSGS, while only modestly increasing the amount of engraftable leukemias, elevate the malignant chimerism levels on average one order of magnitude. The relative myeloid

engraftment is increased 2-3-fold as well (Coughlan et al., 2016; Wunderlich et al., 2010).

A certain time after transplantation the chimerism (percentage of human cells in the mouse blood and bone marrow) is assayed. Higher chimerism argues for higher number or higher activity of stem cells. The initial hematopoiesis is established by progenitors, and to evaluate the contribution of *de facto* HSCs, chimerism is assayed at least four months post-transplant. While this may seem like a long time, several studies have suggested that four months still may not be enough. By introducing unique genetic barcodes in HSCs before transplantation, it was shown that some HSCs lie dormant for near a year before giving rise to progeny (reviewed in (Ema et al., 2014)). Complete evaluation of HSCs can therefore easily prove to a lengthy endeavor.

While the NSG/NSGS is the most widely used, and likely one of the best *in vivo* assays of human hematopoiesis, there at least five major error factors, where several are shared with evaluation of murine hematopoiesis as well. First, the lineages; in effect, we are only assaying hematopoietic progenitors and leukocytes *in vivo*. While progenitors of both the erythroid and megakaryocytic lineage are analyzed, the donor contribution to mature erythrocytes and platelets is not. Second, time; murine hematopoiesis has been proposed to come in waves, where certain HSC clones lie dormant for >1 year. Likely, this is similar for human hematopoiesis and therefore, bone marrow analysis after four-five months might display an incomplete picture. It is of course worth asking whether this risk motivates such a profound delay in research as would be required. Third, location; the contribution of different bones to the transplanted hematopoietic system is vastly different (Rundberg Nilsson et al., 2015). To account for this error, the majority of mouse bones would have to be collected, including the spine and cranium, and not just the tibia, femur and sometimes iliac bones as is usually done. Fourth, the external environment; the environment of immunodeficient mice is quite clean, whereas transplanted human are constantly challenged with foreign microorganisms, even in the clean wards of a hematology clinic. And fifth, the internal environment; it does not matter how many human cytokines are expressed in the murine bone marrow, it will still be murine. Whether there are missing external cues, and the transplanted human cells have to rely on intrinsic regulators only for the establishment of a new blood system, will always be in question.

Despite the drawbacks of *in vivo* mouse models, the majority of results obtained from such studies have been translatable to transplantation into human hosts. It is a matter of whether the question being asked is more academic or practical.

Intertwined with both the *in vitro* and *in vivo* assays is perhaps one of the most useful tools used to investigate the hematopoietic system; flow cytometry. Using flow cytometry, cells can be separated based on three key characteristics; size and

shape, complexity of their cytoplasm, and presence of specific proteins both inside the cell and on the cellular membrane.

An invention that has revolutionized all of life science is next generation sequencing (NGS). Initially, in order to sequence DNA, the region of interest had to be PCR-amplified and only one sequencing reaction could occur in one test tube. With NGS, billions of sequences can be analyzed in parallel, several orders of magnitude cheaper and, more importantly, faster. Further, NGS is not limited to DNA sequencing; the epigenetic and transcription profiles, transcription factor (TF) localization, chromatin accessibility and chromatin interactions can readily be assessed as well using RNA-sequencing (RNA-Seq), chromatin immune-precipitation (ChIP), assay for transposase-accessible chromatin (ATAC-Seq) and Hi-C respectively, the last three naturally combined with NGS as well. While it was initially thought that such an in-depth analysis would make research easier, in reality, it has rather made it harder. The depth and resolution this technology provides, generates a huge amount of data, which requires the skills of an experienced bioinformatician, but also scientific acumen to separate technological artifacts from actual findings.

The combination of flow cytometry with NGS has proven to be quite a powerful toolset. The advance we can expect in the field of NGS in the near future will make it possible to answer several of the most pressing questions. Since virtually all HSPC populations appear to be more heterogeneous on the population level and less plastic on single cell level than was previously thought, optimizing the applications of NGS to suit smaller and smaller input number of cells will likely bring more clarity on this dynamic process. RNA-Seq has already been performed successfully on single cell level, and pilot studies for ATAC-Seq are well on the way. ChIP and Hi-C were originally designed for huge cell quantities, and likely the protocols will have to be changed substantially before such assays can be done reproducibly at the single cell level. Nevertheless, it is likely not more than a few years away, and with it, an even deeper understanding of murine and human hematopoiesis.

4. Regulation of the hematopoietic stem cell

A hematopoietic stem cell, as it resides in the bone marrow, has several different fate options (Figure 2). It can (i) enter quiescence (i.e. G0), where it lies dormant, waiting for an activating signal. (ii) The HSC can go into apoptosis, for instance if it sustains serious DNA damage from radiation exposure or chemotoxic agents. (iii) The HSC can expand if there is a shortage of both HSCs in the body, or if some HSCs are removed as in a donor bone marrow aspiration. (iv) The HSC can differentiate to give rise to mature progeny; presumably their most common role. (v) It can transform to a malignant cell through a series of mutation acquisitions, in effect becoming a leukemic stem cell (LSC), vital in establishment and maintenance of disease, and a critical target of new cancer therapy drugs.

The balance between (iii) and (iv) is a field of intense investigation. It is of particular interest what controls whether the HSC divides symmetrically or asymmetrically. Naturally, the delicate balance of these five different fate options is controlled in a highly complex manner. HSCs are regulated in many ways, by direct interaction with other cells in their niche, endocrine, paracrine and perhaps even autocrine signaling, genetically and epigenetically.

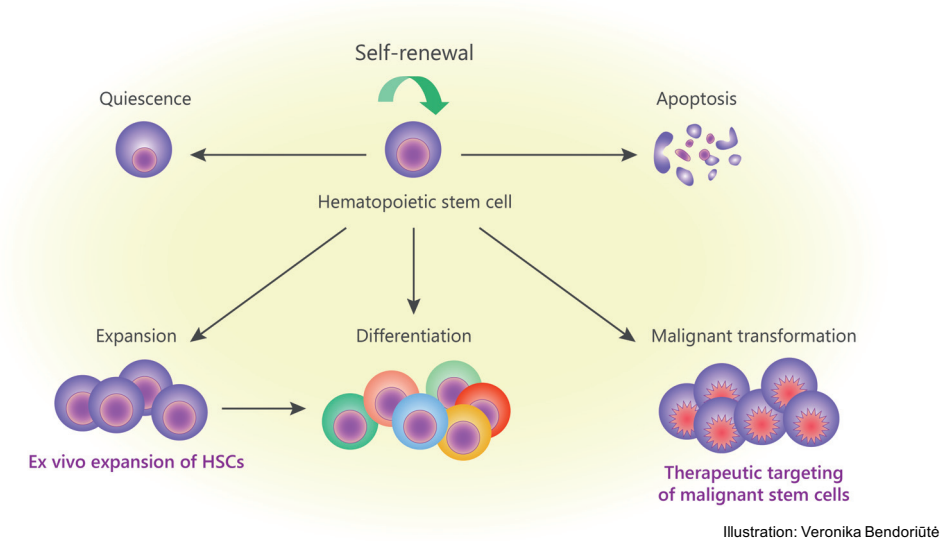

Figure 2. The various cell fate options of the HSC as it resides in the bone marrow.

4.1. Extrinsic regulators

Broadly, extrinsic regulators of HSCs can be divided into two categories; regulators that exert their effects locally in the "HSC niche", and regulators that control HSC behavior from afar, such as the endocrine and nervous systems (Figure 3). The concept of an HSC niche was first proposed by Schofield, in 1978, when he was reviewing a myriad of articles describing the biology and behavior of CFU-S (Schofield, 1978). Since then, the definition of the HSC niche has come to be by anatomy and function (Morrison and Spradling, 2008; Scadden, 2006); a local tissue microenvironment in the bone marrow that regulates HSPC behavior.

In the decades that followed, the key components of the HSC niche have been characterized and visualized. HSCs reside in a complex environment in the bone marrow where they are subject to a wide range of both short-range and long-range regulatory signals. The vasculature in the marrow is composed of sinusoids and arterioles enriched along the bone surface (Nombela-Arrieta et al., 2013). The blood vessels are made up of a single endothelial cell layer surrounded by perivascular mesenchymal stem/stromal cells. Nerve fibers of the sympathetic nervous system run along the blood vessels. The marrow is well vascularized and innervated permitting a quick response and ability to mobilize HSPCs into the bloodstream. There are many secreted factors and membrane bound ligands that regulate HSCs; stem cell factor (SCF), platelet factor 4 (PF4 a.k.a. CXCL4), transforming growth factor beta-1 (TGF-β1), thrombopoietin (TPO) and angiopoietin (ANGPT1) control quiescence. Vascular cell adhesion protein 1 (VCAM-1), CXCL12/CXCR4, fibronectin, hyaluronic acid and several different

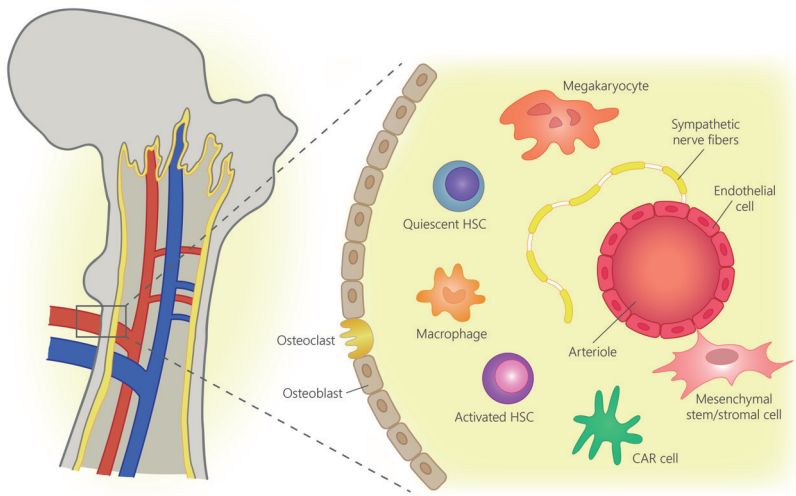

Figure 3. A simplified illustration of the HSC niche in the bone marrow. Illustration: Veronika Bendoriūtė

selectins are all important for HSC homing and anchoring in the niche. Notch ligands, erythropoietin (EPO) and various interleukins are important drivers of proliferation and differentiation (Schepers et al., 2015).

Signals to the HSCs may come from direct cell-to-cell contact, as well as in a paracrine and endocrine manner. The cells in the niche can broadly be divided into two types; essential types and accessory types. The essential types are endothelial cells (EC), mesenchymal stromal cells, also known as mesenchymal stem cells (MSC), and functional, differentiated megakaryocytes (Megs). These three cell types are in direct vicinity of HSCs, and provide direct signals to them, both through secretion of factors as well as by expressing ligands on their cell surface that directly interacts with receptors on HSCs. The accessory cells types consist of osteoblasts (OB); cells that are responsible for building up bone, macrophages, and nerve cells. While these three cell types have an effect on HSCs, they exert their effects through long-range interactions or by an indirect effect; affecting the essential cell types than in turn influence HSC behavior (Frenette et al., 2013; Pietras et al., 2011).

While the concept of an HSC niche is near 40 years old, our understanding of it is still quite limited, and there have been several major controversies in the field, even regarding the actual location of HSCs in the niche. During the last decade, much due to advances in gene technology and especially imaging, the controversies have slowly but steadily begun to be resolved, and with the speed at which research and technology improvement is currently being done, it is likely that the remaining controversies will be solved within the next several years.

The osteoblasts were the first niche cells shown to influence HSPC behavior (Calvi et al., 2003; Zhang et al., 2003), and initially, it was believed that the quiescent, LT-HSCs reside in their immediate vicinity at the surface of the endosteum (Nilsson et al., 2001). This location, furthest away from the blood vessels, was also believed to be hypoxic, where the LT-HSCs dependence on HIF-1α signaling supported this notion (Takubo et al., 2010) along with the fact that the LT-HSCs stained with pimonidazole, indicating hypoxia (Kubota et al., 2008; Levesque et al., 2007). Additionally, transplanted HSCs tend to preferentially migrate to the endosteum (Ellis et al., 2011). Other studies have instead argued that LT-HSCs instead are located in spaces near the vasculature (the so called perivascular spaces) (Kiel et al., 2005; Kunisaki et al., 2013).

Due to better imaging resolution and the ability to use the same markers in confocal microscopy as for flow cytometry, this conflicting finding has almost been completely resolved. It is now believed that the majority of LT-HSCs are in fact perivascular, and only 20% are located within a few cell diameters (10-20μm) of the endosteum (Kiel et al., 2007; Kiel et al., 2005; Lo Celso et al., 2009; Nombela-Arrieta et al., 2013). However, HSCs are found in the trabecular region

of the bone marrow, arguing, at least, for an indirect regulation by factors on the bone surface. Selective depletion of osteoblasts from otherwise unperturbed bone marrow had little effect on HSCs though, rather affecting progenitors, particularly of the B-cell lineage. The trabecular bone itself, formed by osteoblasts, has been shown to be critical for hematopoiesis. It is therefore more likely that osteoblasts play a role in the formation of the HSC niche itself, rather than directly regulating HSCs (Chan et al., 2009; Sacchetti et al., 2007; Zhou et al., 2010).

While it is clear that HIF-1α is important for HSC maintenance, the signalling from this transcription factor does not necessarily have to be induced by hypoxia. Indeed, cells adjacent to pimonidazole-staining LT-HSCs remained unstained, and another imaging study using a nanoprobe instead of pimonidazole, found that oxygen tension was actually low around the sinusoids and high at the endosteum (Nombela-Arrieta et al., 2013; Spencer et al., 2014). Since most LT-HSCs as well as progenitors appear to be located around the vasculature, and activated progenitors rely on oxidative phosphorylation to meet their high energy demands rather than just glycolysis, as is preferred by quiescent HSCs, it is more likely that it is the consumption of oxygen due to active hematopoiesis that causes a hypoxic environment, rather than there being an oxygen gradient caused by diffusion barriers.

The data acquired during attempts to solve the controversies regarding the exact nature of the contributory role of cells from the osteoid lineage indicates that different progenitors have their own "sub-niches". In addition to osteoblasts being important for development of B-cells, it has been shown that macrophages are crucial for erythroid development (Chow et al., 2013). More experiments are required to understand these relationships.

Non-hematopoietic cells in the niche regulate HSPCs both through membrane-bound and soluble ligands as well as cytokines. ECs express CXCL12, essential for homing of HSPCs to the bone marrow, SCF, various Notch ligands and E-selectin (Butler et al., 2010; Winkler et al., 2012). MSCs also express SCF and CXCL12, a particular subset of MSCs termed CXCL12-abundant reticular cells (CAR cells) express CXCL12 approximately 100 times higher than ECs (Ding and Morrison, 2013). There exist several other different subsets of MSCs, together controlling a major part of HSC biology including trafficking, relocalization in the niche and cell cycle (Ding et al., 2012; Greenbaum et al., 2013; Mendez-Ferrer et al., 2010; Omatsu et al., 2010). Megs secrete quiescence-inducing factors; CXCL4, TGF-β1, and TPO, but also factors that induce HSC proliferation and expansion in response to stress such as FGF1 (Bruns et al., 2014; Nakamura-Ishizu et al., 2014; Zhao et al., 2014b). Osteomacs, bone associated macrophages, and osteoclasts, macrophages specializing in bone resorption through secretion of protons and collagenases, control HSCs indirectly by crosstalk with MSCs and

remodeling of bone. Lastly, sympathetic nerve fibers activate the β3-adrenergic receptors expressed on MSCs and OBs in a circadian fashion. This leads to downregulation of CXCL12, leading to the periodic mobilizations of HSPCs into the bloodstream (Asada et al., 2013; Mendez-Ferrer et al., 2008).

Findings in the human HSC niche have been acquired through primarily two different research strategies; histological sectioning of both benign and leukemic bone marrow biopsies, and *in vitro* co-culture of human HSPCs with various cells known to be part of the niche, assessing their effect on stem cell proliferation and maintenance. The results are quite similar to murine studies; 86% of $CD34^+$ cells are found around perivascular MSCs (Flores-Figueroa et al., 2012), and the more undifferentiated $CD34^+CD38^-$ population is enriched around the trabeculated bone areas (Guezguez et al., 2013). Several studies have confirmed the importance of MSCs for human HSCs as well, where co-culture was shown to enhance their *in vivo* repopulating ability (Li et al., 2014; Pinho et al., 2013; Taichman, 2005). In an effort to better understand and model the human hematopoietic niche, several groups are developing advanced 3D culture systems and mouse models containing ectopic human BM-microenvironments (Chen et al., 2012; Groen et al., 2012; Leisten et al., 2012; Raic et al., 2014; Sharma et al., 2012).

Cytokines and interleukins are an essential regulatory element of the hematopoietic (and the immune) system. Generally, pro-inflammatory cytokines, such as tumour necrosis factor α (TNF-α), IL1β, IL3 and IL6 amongst others, are positive regulations of HSC differentiation and negative regulators of quiescence. This is seemingly intuitive since inflammation requires activation of the hematopoietic system increasing its output, particularly in the myeloid lineage. On the other hand, SCF and TPO are two cytokines promoting HSC maintenance and quiescence and are quite often used in *in vitro* culture systems of both murine and human HSCs. Quite interestingly, the effects of TPO seem to be somewhat different *in vitro* vs. *in vivo*. TPO enhances both survival and proliferation of HSPCs *in vitro*, while *in vivo*, TPO is rather inhibiting apoptosis through by suppressing p53 (Borge et al., 1996; Ema et al., 2000; Keller et al., 1995; Li and Johnson, 1994; Pestina et al., 2001; Sitnicka et al., 1996; Yoshihara et al., 2007). Naturally, TPO is a regulator of megakaryocytes, and it is quite possible that TPO also regulates HSPCs directly and indirectly, the latter through modification of niche Megs. Further studies will be required to truly map out the nature of this interaction.

During the last decade, it has become more and more appreciated that the HSC niche is an active contributor and even an initiator of hematopoietic neoplasms. The HSC niche has lately also become a key player in the field of stem cell aging, rejuvenation and anti-aging medicine (Neves et al., 2017; Wong et al., 2015). Linking these three processes together is inflammation, a strong, and context

dependent, regulator of HSC activity and fate. The three central inflammatory cytokines; IFN, G-CSF and IL-1 act both as stimulators of HSC proliferation and inducers of myeloid differentiation. Interestingly, the HSC proliferation is transient; after certain time, even if the pro-inflammatory cytokines are still present, the HSCs return to a quiescent state. The increased proliferation is caused by suppression of quiescence-inducing TFs such as Foxo3a, and stabilization of the proto-oncogene c-Myc. Differentiation induction can be caused by activation of multiple signaling pathways, however, the majority of them seem to converge on nuclear factor kappa B (NF-κB)-dependent PU.1 activation (Ehninger et al., 2014; Essers et al., 2009; Pietras et al., 2014; Pietras et al., 2016; Schuettpelz et al., 2014; Zhao et al., 2014a).

Proliferation and differentiation, induced by inflammatory cytokines, including IFNs, IL-1, TLRs and TNF, have been shown to impair the self-renewal of HSCs (Baldridge et al., 2010; Herman et al., 2016; Matatall et al., 2014; Takizawa et al., 2017). These results however, have been derived from transplantation studies, and while quite relevant for BMT (see next chapter), it is unclear whether inflammation causes HSC exhaustion and/or BM failure in a steady-state context, devoid of chemotherapy or infection (Matatall et al., 2016).

While inflammatory cytokines seem to impair HSC self-renewal in the adult system, during development, they rather function as positive regulators of definitive hematopoiesis. Here, G-CSF, IL-1, IL-3, IFN-γ , and TNF, activate NF-κB and Notch1, driving the specification of HSCs from hemogenic endothelium (Espin-Palazon et al., 2014; Kim et al., 2014; Orelio et al., 2008; Robin et al., 2006; Stachura et al., 2013). Interestingly, mice with deficient NF-κB, subjected to antibiotic therapy during pregnancy or kept in very sterile environments during development have an impaired hematopoiesis and smaller myeloid compartments in particular (Espin-Palazon and Traver, 2016; Grossmann et al., 1999; Josefsdottir et al., 2017; Khosravi et al., 2014). Possibly, on a general level, the inflammatory signals are required to prime the hematopoietic system, to ensure it develops properly and is able to respond adequately and efficiently to address the acute needs during the lifespan of the organism (Pietras, 2017).

Inflammation is usually an acute response to address a certain acute need in the body, an infection for instance. In the case of the inflammation not resolving properly, it becomes chronic, with serious consequences for homeostasis, tissue function, and even life span. Several autoimmune and metabolic diseases cause increased levels of chronic inflammation, and so does aging. Apart from the effect of aging on blood cell composition, aging is also associated with abundance of inflammatory cytokines (including IL-1, IL-6 and TNF), a combination also known as the senescence-associated secretory phenotype (Coppe et al., 2010). SASP cytokines are likely produced by aged BM cells, and we know from

previous studies of their involvement in various hematological malignancies (Orjalo et al., 2009). Some components of the chronic inflammation network are even attributed as key drivers in certain cancer states (Rambaldi et al., 1993; Stifter et al., 2005; Welner et al., 2015). It is not yet known what effects the SASP has on HSCs, but we do know that inflammation together with endogenous DNA-repair defects contribute to a preleukemic state and dysregulation of the BM niche (Walter et al., 2015; Zambetti et al., 2016). Targeting old age itself could therefore perhaps be a quite plausible therapeutic, or preventative, strategy against hematologic (and other) malignancies (Fang et al., 2017; Rhyasen et al., 2013).

4.2. Intrinsic regulators

The identification of intrinsic regulators has been pursued through different techniques in mice and humans. In the mouse, a large fraction of now known regulators were identified through classic reverse genetics knockout studies, where knockout of a gene causes decreased HSC activity (positive regulator) or increased HSC activity (negative regulator). Considerable effort has been devoted to characterization of murine knockout models. In human cells on the other hand, many discoveries were made during the quest for *ex vivo* expansion of HSPCs for transplantation purposes. Here, the focus has been not only on proliferation, but on improved homing to the bone marrow as well. On a general level, HSCs are governed by pan-cellular mechanisms; cell cycle regulation, stress- and damage response to DNA and organelles, metabolic activity, chromosome conformation, transcription factors and miRNAs as well as more HSC-specific genes and pathways (Domen et al., 2000; Folmes et al., 2012; Han et al., 2010; O'Connell et al., 2010; Ooi et al., 2010; Pietras et al., 2011; Sauvageau et al., 2004). Efforts to expand engraftable human HSCs have yielded a hit list of a wide diversity.

Several genetic pathways, critical during embryonic development, also control the fate of HSPCs during adult life. The Homeobox (Hox) family of transcription factors (controlling body axis segmentation during development) have been extensively studied in murine HSCs, where overexpression of HoxA9 and HoxB4 in particular have shown drastic increases in engraftable murine HSCs. HOXB4 has been found to be somewhat translatable to the human system (Amsellem et al., 2003; Antonchuk et al., 2002; Krosl et al., 2003). Overactivation of the Notch signaling pathway causes similar results, with a near 6-fold increase in engraftable $CD34^+$ cells in NSG mice (Delaney et al., 2010; Delaney et al., 2005; Ohishi et al., 2002).

It has been somewhat more difficult to ascertain the precise role of Wnt signaling, possibly due to there being several receptor types with a complex interaction pattern with their various ligands, or as in the case for TPO, a seemingly different

effect and requirement of Wnt *in vitro* vs. *in vivo*. Nonetheless, active Wnt signaling enhances self-renewal and proliferation of HSCs *in vitro* (Austin et al., 1997; Reya et al., 2003; Willert et al., 2003), and inhibition of GSK-3β (a negative regulator of Wnt signalling) enhances *in vivo* reconstitution ability of both murine LSK cells and human $CD34^+$ cells (Ko et al., 2011; Trowbridge et al., 2006). While studies of an isolated signaling pathway yields important insights, it must be kept in mind that many pathways likely interact with each other. For instance, Wnt signaling, while having a HSC promoting effect in its own right, has also synergistic effects with other central signal transduction pathways including Notch, PKB and mTOR (Duncan et al., 2005; Huang et al., 2012; Perry et al., 2011). Furthermore, inhibition of GSK-3β seems to contribute to leukemic development, partly by affecting Akt and mTOR in addition to Wnt (Guezguez et al., 2016).

It is a general consensus that HSCs are usually kept dormant and quiescent in their niche during steady state. Excessive cell cycling induced by loss of cyclin-dependent kinase inhibitors (CDKN) is generally associated with stem cell exhaustion (Pietras et al., 2011). Interestingly, lentiviral knockdown of CDKN1A (p21), while pushing the HSCs into cycle, causes a relative expansion of the HSC pool (Stier et al., 2003), contrary to the effect observed when depleting other members of the same (CDKN1; p21, p27 and p57) or related (CDKN2; p15, p16, p18 and p19) families of cell cycle inhibitors, which instead all cause exhaustion. The complete mechanism behind this effect is not entirely understood though.

Expansion of umbilical cord blood (CB) derived HSCs has been a major goal of hematopoietic transplantation medicine for decades. Several important human HSC regulators have been identified due to these efforts.

By screening for a stimulatory effect on the human $CD34^+$ population obtained from the mobilized peripheral blood of healthy donors using a very wide range of small molecules Boitano et al. identified StemRegenin1 (SR1) as a potent activator of human HSC activity, increasing it near 17-fold as measured by transplantation into NSG mice (Boitano et al., 2010). This effect was attributed to binding and antagonism of the aryl hydrocarbon receptor (AHR). AHR is perhaps best known as the receptor for dioxin, known cancer-inducing mutagen and environmental toxin.

Several subsequent studies have tried to uncover the mechanism behind this dramatic increase. However, given that AHR is a receptor that primarily influences cell function through its effects on transcription, this has proven to be quite tough. As is custom, this question has been addressed using traditional mouse knockout studies. The results are somewhat conflicting depending on which part of AHR was deleted and which mouse strain was used but overall; homozygous loss of AHR results in increased levels of phenotypic LSKs and LT-

HSCs but at the expense of functionality, resulting in decreased chimerism levels where the AHR deficient hematopoiesis resembles aged hematopoiesis and even myelodysplasia and MDS (Singh et al., 2014). Interestingly, this points to a dual and dose-dependent role of AHR signaling. While partial antagonism stimulates proliferation of engraftable HSCs, partly through modulation of the endosteal BM niche (Unnisa et al., 2016), knockout results in decreased reconstitution and development of malignancy, and agonism of AHR supports malignant development as well (Murray et al., 2014). The case of AHR nicely illustrates the difficulty of delineating the mechanism of action and even the identification of upstream and downstream targets; the heterogeneity of HSPCs, different experimental methods and dose sensitivity all affects the results and their interpretation.

Prostaglandin E2 (PGE2) is another molecule frequently mentioned in the context of HSPC expansion. Prostaglandins are mostly known for their role in the immune response, increasing blood flow and capillary permeability in response to inflammatory stimuli. While initially discovered in the murine system, where stimulation by PGE2 increased both self-renewal and homing (by upregulation of CXCR4, downregulation of caspase 3, and modulation of Wnt-signalling pathway) (Frisch et al., 2009; Goessling et al., 2009; Hoggatt et al., 2009), similar enhanced function has been demonstrated for human $CD34^+$ HSPCs as well (Goessling et al., 2011).

Epigenetic regulation, (the concept of modifying genes by methylating DNA or methylating and/or acetylating histones rather than changing the actual DNA sequence,) is another critical pan-cellular mechanism of gene regulation. In the context of stem cell biology, an inverse correlation between chromatin accessibility (euchromatin) and cell differentiation, has recently become more appreciated and established (Ugarte et al., 2015). This was initially believed to give embryonic and hematopoietic stem cells in particular their plastic, pluri/multipotent state, however, the recent discoveries described above ("2.3. Biology of HSPCs") regarding lineage restriction in LT-HSCs have brought some doubt in this regard. Nonetheless, modulation of epigenetic modification has also proven to be a viable strategy to instruct cell fate.

Adding the well-known histone deacetylase (HDAC) inhibitor valproic acid (VPA) has been shown to enhance the proliferation and both murine and human HSPCs *in vitro* (Bug et al., 2005; De Felice et al., 2005; Seet et al., 2009). The polycomb repressor complexes (PRCs) methylate histones at specific lysine residues, thereby modulating gene expression. PRC1 and PRC2 appear to have an opposing role in HSPCs, where PRC1 promotes self-renewal while PRC2 promotes differentiation (Majewski et al., 2010). Modifying core or accessory proteins of these two complexes is therefore likely a feasible strategy to influence

HSPC fate and is the topic of **paper II** presented in this thesis. Lysine-specific demethylase 1 (LSD1) is yet another indispensable regulator of differentiation as well as HSC activity in murine HSPCs, whose effect is quite context dependent (Kerenyi et al., 2013). Fine tuning the activity of this epigenetic regulator will likely be a feasible strategy to influence HSPC fate.

The effect of epigenetic modifiers is often not as clear cut as in the examples above. Azacytidine and decitabine (RNA and DNA-based analogues of cytidine), two hypomethylating agents (HMA) nicely illustrate this. Used in the treatment of various hematological malignancies, the mechanism of action seems to be opposite of the expected; the hypomethylation caused by this drug releases the differentiation block observed in AML and MDS rather than promoting HSC self-renewal (DNA methylation and differentiation is normally associated with gene silencing, it would therefore be more plausible to expect decreased differentiation in the hypomethylated state and not the opposite). HMAs have several mechanisms of action though, and it is also not entirely clear which genes get hypomethylated. Indeed, it is possible that the differentiation inducing genes are more sensitive to HMA than others. (Stresemann and Lyko, 2008).

Lastly, there are several orphan regulators and "expanders" of HSPCs; compound and molecules with an often profound effect on HSPC expansion, but where a clear mechanistic target has not been identified. A good example of this is the pyrimidoindole derivate UM171 that expands human $CD34^+$ cells near 250-fold *in vitro* and over 13-fold *in vivo*, but while gene expression studies clearly identify differentially expressed genes, a mechanism of action is still not clearly identified (Fares et al., 2014). In our own group, we have identified multiple shRNAs with off-target effects showing a profound effect on human HSPC expansion *in vitro* and *in vivo*, but where the true target could not be identified despite rigorous attempts (Ali et al., 2009; Baudet et al. unpublished data; Galeev et al. unpublished data).

To summarize, we know that developmental signaling pathways and transcription factors (Notch, WNT, HOXB4), pyrimidoindole derivatives (SR1, UM171, tranylcypromine), inflammation modulators (PGE2, TNF-α, NF-κB) and epigenetic modifiers (acetylation and methylation modifiers) all have an effect on human HSC function. Genetic regulators of HSPCs identified using RNA interference screens are described below in chapter 3.3 in the last part of the thesis.

MALIGNANT HEMATOPOIESIS

1. Introduction to malignant hematopoiesis

Malignancy, or cancer, can easily be defined as cells that have lost their cell cycle control and boundaries in the tissues. Thus, they show excessive proliferation, invasion, and metastasis. The organ infiltration shuts the organs off directly (liver metastases causing hepatic failure, bone marrow invasion causing pancytopenia etc.) or indirectly through hormonal effects, the so-called paraneoplastic syndrome. Inevitably, through these mechanisms, cancer leads to multiorgan failure and death.

In solid-state tumors, there is usually a sequential accumulation of mutations that results in increased proliferation, decreased differentiation, resistance to apoptosis, loss of boundaries, invasion into other tissues, and finally metastasis. This malignant transformation requires many mutations to occur, and often there are chromosomal translocations or outright aneuploidy (chromosome loss or gain, resulting in abnormal amount of genetic material in the malignant cell).

Blood, the largest of the very few "liquid" organ systems in the body, has a different dynamic of malignant transformation. First, significantly less mutations or chromosomal translocations are required for development of a severe hematopoietic cancer (leukemia). Often, mere two mutations or a single chromosomal translocation is enough. Possibly, this is due to the inherent high proliferative state of blood cells; less is required for malignant transformation. Second, mutations in the "classic" cancer genes are less common (such as TP53, RB1, ATM, BRCA1/2), rather mutations in epigenetic regulators, chromatin modifiers and the spliceosome machinery are prominent in the genetic landscape (Welch et al., 2012). Third, not all leukemias show a clear causative mutation, in some cases, the leukemic driver is not known.

Leukemias are usually categorized based on how quickly they develop (acute vs. chronic), and which hematopoietic lineage affected (myeloid vs. lymphoid), in effect generating four major groups; acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL) and chronic lymphogeneous leukemia (CLL). In the case of leukemia, the myeloid lineage includes malignancies of the erythroid and platelet lineages as well. In about 10% of cases, the leukemia contains cancerous cells of different lineages and is then called mixed-lineage-leukemia (MLL). The common denominator for leukemias is their cell of origin, all leukemias develop from undifferentiated cells; either stem

cells or progenitors. Cancers of mature B-cells and mature T-cells are naturally also disorders of the hematopoietic system, but are traditionally called lymphomas. They have different pathophysiology and presentation and are thus treated fairly differently from leukemias and will not be covered here. It should be pointed out however, that BMT is a curative treatment option for lymphomas as well. Due to complications and the course of the disease, this option is more rarely pursued in the context of lymphoma treatment.

The hematopoietic system also harbors several pre-malignant states. Despite variation in the severity of symptoms, the pre-malignant states have an increased risk of further on developing into a full-blown cancer. The pre-malignant states include the myeloproliferative neoplasm (MPN) consisting of polycythemia vera (PV), essential thrombocytosis (ET) and myelofibrosis (MF). CML can also be categorized as a pre-malignant state, that in the absence of therapy leads to a blast crisis that closely resembles AML. Monoclonal gammapathy of unknown significance (MGUS) is perhaps the most classic premalignant state, with an annual risk of 1% of developing multiple myeloma (MM).

In the context of AML and MDS (see below), it has recently been shown that these two diseases are often preceded by "clonal hematopoiesis of indeterminate potential" (CHIP). Clonal hematopoiesis is characterized by clonal expansion of the HSC pool; i.e. there are several-fold fewer HSCs giving rise to mature cells as compared healthy hematopoiesis. These HSCs have thus undergone clonal expansion. The 115-year-old lady mention in the beginning most certainly had CHIP. Interestingly, it is the same mutations that cause CHIP as AML and MDS. While the complete significance of this premalignant state has not been completely delineated, it is likely that we will be able to use CHIP as a quantitative risk marker in the future (Jaiswal et al., 2014).

Finally, there is myelodysplastic syndrome (MDS), an HSC disorder characterized by increased HSC proliferation with subsequent exhaustion resulting in cytopenias in one or any combination of the three hematopoietic lineages. While this disease is characterized by underproduction of both progenitors and mature blood cells, often displaying decreased cellularity in the bone marrow, just as for MPN, there is a significant risk of the phenotype "reversing" and transformation into AML, effectively making MDS a pre-malignant state also.

It has been known for many years and is widely accepted that in the context of hematopoietic disease, particularly cancers of the blood system (leukemia), the cell of origin is an undifferentiated hematopoietic cell, either an HSC or shortly downstream. In the context of the niche, there two fundamental mechanisms of niche contribution to hematopoietic disease; altered signal interpretation and niche remodeling (Schepers et al., 2015). After the discovery that CML progenitors have altered adhesion properties in 1987 (Gordon et al., 1987), more recent studies have

shown that (i) leukemic cells can "hijack" the vascular niche space by responding more strongly to homing signals than healthy HSCs, thereby outcompeting them (Schepers et al., 2013; Sipkins et al., 2005). (ii) leukemic cells become less sensitive to Notch and TGF-β, signals that normally limit primitive cell expansion and myeloid differentiation (Krause et al., 2014; Santaguida et al., 2009). (iii) Leukemic cells can remodel the niche, including stromal cells, osteoblasts and nerve fibers, to support LSCs over healthy HSCs. LSC are also less dependent on survival signals from the niche compared to healthy HSCs (Arranz et al., 2014; Hanoun et al., 2014; Schepers et al., 2015). (iv) Contrary to the old dogma where leukemia is initiated through mutation acquisition in hematopoietic cells; mutations in non-hematopoietic BM cells alone have shown to be enough to cause leukemias. Errors in NF-κB, Notch and Wnt signaling as well as mutations in the miRNA processor Dicer1, is enough to cause both an MPN-like and an AML-like disease in otherwise healthy, normal HSPCs (Kim et al., 2008; Raaijmakers et al., 2010; Rupec et al., 2005; Walkley et al., 2007a; Walkley et al., 2007b). It is however, not definitely proven whether this occurs in humans, and while sequencing of biopsy samples indicates that this could indeed be the case, the number of studies is so far limited. Kode et al. showed that human HSPCs from AML patients had upregulated Notch signaling as a direct consequence of Wnt mutations in the BM stroma (Kode et al., 2014), and there are major discrepancies observed between the mutational landscape of malignant HSPCs and their stroma (Blau et al., 2007; Kastrinaki et al., 2013). However, given that the biopsies are taken at the point of disease, it is hard to ascertain which genetic aberration is the actual initializing event. A study including 80 patients with AML, MDS or MPN showed that near 40% had mutations in the BM stroma in β-catenin and RUNX2; mutations that were not present in the actual hematopoietic malignant cells. Due to the time of sample collection, it is yet unclear whether the initiating mutational event occurred in cells of hematopoietic or of non-hematopoietic origin. (v) Regardless of origin and causality, mutations can happen in all cell types in the BM niche; ECs, OBs, OBC, MSCs, and nerve cells. Separately, or together, they can all contribute to hematopoietic disease (Arranz et al., 2014; Hanoun et al., 2014). These discoveries open up a very interesting therapeutic opportunity in targeting not only the leukemic cells, but the dysfunctional and/or leukemic niche as well.

2. Acute myeloid leukemia

2.1. Epidemiology and pathogenesis

AML is the most common leukemia in adults, where it accounts for around 80% of leukemias (Yamamoto and Goodman, 2008) with an incidence of 3-5 new cases per 100 000 people per year (Siegel et al., 2017). While AML can occur in young adulthood, and even in children, its incidence strongly increases with age (>12 in people over 65 years of age). Contrary to CML, there are few inhibitor drugs on the market for AML, although clinical trials have been initiated. The cornerstone of AML treatment is BMT, but the severe intensity and stress of this therapy is poorly tolerated in older individuals, especially those above the age of 70, where this disease has its highest prevalence. Because of this, and the aggressive nature of this leukemia subtype, the prognosis for the older patients remains dismal; near 70% will die of their disease within the first year after diagnosis (Meyers et al., 2013), while advances have significantly improved prognosis of younger patients with AML. In approximately 60% of AML cases, the karyotype is normal, with the remaining 40% showing various levels of aneuploidy (Hiddemann et al., 1986).

While the majority of AML cases are *de novo*, AML has the unique property of being an “end stage point” for several other hematological conditions. While ALL and CLL are isolated diseases in their own right, and AML could start *de novo*, it can also develop from myelodysplastic syndrome (MDS), from CML as well as from the MPNs; PV, ET and MF (Sill et al., 2011).

Regardless of origin, the pathology of AML involves proliferation of immature myeloid cells in the bone marrow, the blood, and occasionally in other organs. This massive proliferation results in a huge number of dysfunctional cells that occupy the bone marrow, decreasing the output of healthy and functional blood cells through multiple mechanisms as described above. If left untreated, death occurs merely a few months after developing the disease, from invasive infections, bleeding, emboli, or disseminated intravascular coagulation causing multiorgan ischemia (Figure 4). AML is classified based on lineage/phenotype of the leukemic cells as well as genetic alterations, in effect subdividing AML into three prognostic categories; favorable, intermediate and adverse (Dohner et al., 2010; Mrozek et al., 2012).

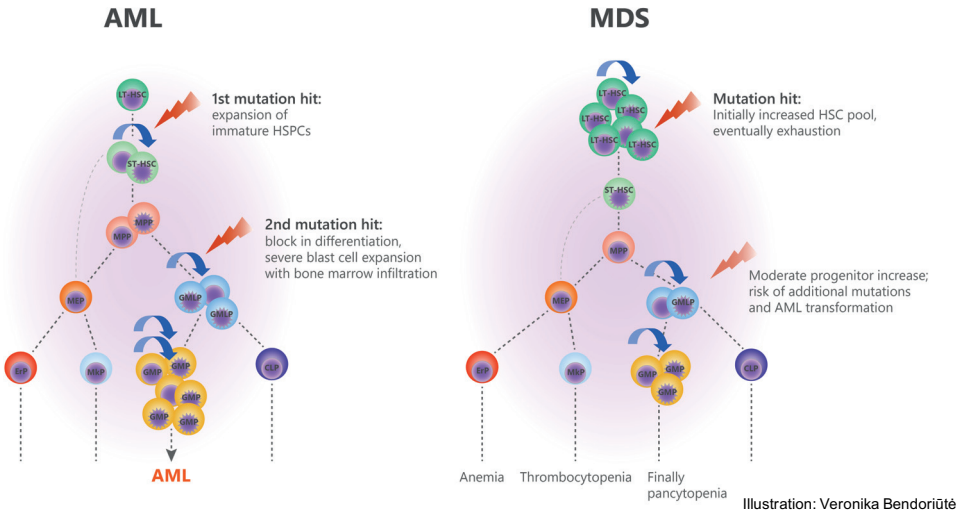

Figure 4. Schematic illustration of the development of AML and MDS.

2.2. Treatment

Currently, therapy for AML consists of several stages. First, induction therapy is given, a treatment designed to decrease the amount of the leukemic cell burden several orders of magnitude. Induction therapy usually consists of cytarabine (a nucleoside analog leading to apoptosis) and an anthracycline (DNA alkylator and topoisomerase II inhibitor, thus interfering with both replication and transcription).

While this therapy is often enough to drastically reduce disease burden, relapse will inevitably follow if the therapy is discontinued (Estey, 2014; Gong et al., 2015; Li et al., 2015). This has recently been attributed to the presence of leukemic stem cells (LSCs) that contrary to their progeny, are much more resistant to chemotherapy (Pollyea et al., 2014).

To minimize the risk of relapse, induction therapy is followed by consolidation therapy, which can be either additional chemotherapy (additional nucleoside analogs and anthracyclines), or bone marrow transplantation (BMT). While it is unclear which of these two forms of consolidation is a better option for AML with a favorable prognosis, BMT is significantly better for patients with intermediate risk and clearly better for patients with adverse risk (Koreth et al., 2009; Yanada et al., 2005). This is likely due to the graft-vs-leukemia (GvL) effect, i.e. cells from a donor are considerably more effective at eliminating the remnants of leukemia including the LSCs than conventional chemotherapy. Indeed, patients experiencing graft-vs-host disease (GvHD) have a higher risk of relapse since GvHD is treated with immunosuppressants, attenuating the GvL at the same time (Kolb, 2008).

BMT however, carries a risk in its own right, given that the patient's own hematopoietic system needs to be destroyed before donor bone marrow can be transplanted. Apart from the overall systemic toxicity of chemotherapeutic agents, this procedure also results in a several day-long period where the patient does not have a functional immune system, and despite prophylactic antibiotics, that after all work together with the immune system and not independently, can prove fatal, particularly in older patients. To improve survival for older patients with AML (>65), it is therefore pivotal to either make BMT a safer procedure, or design targeted therapies (targeting the leukemia or the leukemic niche) to ensure eradication of LSCs and prevent relapse upon discontinuation of chemotherapy.

However, given the genetic diversity of AML, developing targeted therapy beyond Vitamin A derivatives (described in detail in part 5 below) has proven hard. There are several drugs in clinical trials; FLT3 inhibitors sorafenib, midostaurin, quizartinib and crenolanib, IDH1 and IDH2 blockers ivosidenib and enasidenib, and monoclonal antibodies such gemtuzumab (targeting CD33) all show good results, particularly in patient with an unfavorable cytogenetic and genetic mutation profile, but are restricted to patients harboring these particular genetic mutations. Chimeric antigen receptor (CAR) T-cells, while quite successful in lymphoblastic leukemias and some lymphomas, have so far not been successful in AML due to very serious side effects (targeting CD33 results in profound myeloid cytopenia, and other appropriate antigens have so far not been identified).

3. Myelodysplastic syndrome

3.1. Epidemiology and pathogenesis

Myelodysplastic syndrome, while it shares many mutations with AML is fundamentally a different disease. Whereas in AML the bone marrow produces to many cells, patients with MDS produce too few. The incidence of MDS is similar to AML with 3-4 cases per 100 000 people per year, but an even greater skewing towards old age, with an incidence of 30 in people above the age of 70 (Sekeres, 2010). MDS has a very variable prognosis with median survival, much more so than AML, ranging from 5 years to 6 months depending on karyotype, with an overall median survival of 30 months (Germing et al., 2013; Greenberg et al., 2012).

The mutation spectrum of MDS is similar to that of AML, however, around 50% of cases show MDS-specific, cytogenetic abnormalities (Gangat et al., 2015; Schanz et al., 2012). Clinically, MDS is characterized by cytopenias, either in one

lineage, or in several. Interestingly, the cytopenias have no correlation to the cellularity in the bone marrow, which can be hyper- normo- or hypocellular (Figure 4). From a therapeutic perspective, management of MDS is fraught with challenges and complications arising from the cytopenias; anemia (with tiredness, dizziness, headache and malaise in milder cases and myocardial infarction in more severe), thrombocytopenia (with excessive bleeding, particularly from the mucosa, in effect causing a secondary anemia), and leukopenia resulting in frequent and recurrent infections. Furthermore, there is a 30% risk of a leukemic transformation, where the MDS transforms into secondary AML (sAML) that responds much more poorly to induction chemotherapy than a *de novo* AML (Greenberg et al., 2012; Malcovati et al., 2007; Sekeres, 2010).

3.2. Treatment

Due to the nature of MDS, the only curative therapy is BMT. However, the strong prevalence skewing towards older individuals with MDS makes the majority of patients ineligible for this therapy. Instead, therapy is focused on management of the symptoms; erythropoietin (EPO) injections or transfusions in patients not responding to EPO, platelet transfusion when these reach a critical threshold and prophylactic antibiotics and/or myeloid stimulating factors such as G-CSF or its synthetic analog filgrastim. A smaller group of patients respond to hypomethylating agents, such as azacytidine or decitabine. Lenalidomide (originally used for treatment of multiple myeloma) could be used to reduce transfusion dependence in patients with del5q MDS. Danazol and ATG can also be used to treat anemia in some patients (Chabannon et al., 1994; Greenberg et al., 2009; List et al., 2006; Young et al., 2006).

There are a number of drugs in clinical trials showing encouraging results; romiplastim and eltrombopag decreasing bleeding and platelet transfusion dependence and sotatercept and luspatercept achieving the same for anemia (Giagounidis et al., 2014; Komrokji et al., 2018; Oliva et al., 2017; Platzbecker et al., 2017), but not only are they effective in a small subgroup of patients, these therapies are for all practical purposes palliative as well; despite some patients with low-risk MDS living with their disease for years, decades even, the majority become non-responsive to the aforementioned therapies, suffer serious side effects from the tranfusions and/or the disease evolves into a sAML. Other examples of drugs in the development pipelines are the histone deacetylase inhibitors mocetinostat och pracinostat, and IDH1/2 inhibitors rigosertib and volasertib, all sharing the same limitations as the approved drugs (Gangat et al., 2016). Since none of the approved drugs for treating MDS are curative (Fenaux et al., 2009;

Kantarjian et al., 2006; List et al., 2005), MDS is perhaps in an even bigger need of novel, targeted therapies than AML.

4. Differences between AML and MDS

4.1. Disease development

Seemingly there are more similarities than differences between AML and MDS. One possible explanation behind the observed different phenotypes behind these two diseases is where in the hematopoietic hierarchy the mutation occurs. MDS is considered a pure "HSC-disease". AML on the other hand, was initially thought to arise more downstream, at the progenitor cell level, with little role of HSCs. However, more recent studies have brought doubt to this theory, instead arguing that the initiating mutations do indeed occur in the HSCs, often giving rise to the clonal hematopoiesis that is observed before the development of *de facto* AML. Eventually, should an additional detrimental mutation occur in a more downstream progenitor (such as the GMP), AML will ensue (Jan et al., 2012). AML caused by fusion genes/chromosomal translocations on the other hand, is likely initiated at the level of GMP. Several studies have clearly shown the relative ease with which AML can be induced upon introduction of a leukemic fusion protein into healthy GMPs. An interesting side note is that LT-HSCs on the other hand, seem to be protected from fusion protein-induced AML (Ugale et al., 2014). The susceptibility of GMP for fusion-protein AML could be due to their shown ability to activate a self-renewal program (Herault et al., 2017). If this program is not turned off properly during differentiation, cancer will likely be the result. It was previously thought that LT-HSCs are somewhat protected from DNA damage due to their quiescent nature and low metabolic rate however, this view has changed and it has now become fairly established that mutations accumulate in LT-HSCs throughout life even without the presence of exogenously introduced DNA damaging agents (such as chemotherapeutic drugs) and regardless of cell cycle status (Beerman, 2017). LT-HSCs could have a strong demand on the integrity of their genome though, or are simply not actively cycling enough for leukemia to develop.

4.2. Mutation landscape

On a genetic level, while many mutations are shared between these two diseases, there are notable differences. Interestingly, out of the three most common

mutations in AML (FLT3, NPM1 and DNMT3A), only DNMT3A is shared with MDS. Likewise, the most commonly mutated gene in MDS; SF3B1, a component of the spliceosome machinery, is not observed in AML while other spliceosomal mutations are. Overall, mutations in AML and MDS can be grouped into several broad categories; epigenetic regulators, spliceosomal machinery, cohesin, DNA processing enzymes, transcription factors and known tumor suppressors (Papaemmanuil et al., 2013; Welch et al., 2012). One of the most common mutation (if not the most common one) in AML is over activation of NPM1, a protein involved in ribosome biogenesis, nucleolar trafficking and G-quadruplex binding. However, this mutation is rare in MDS. Conversely, mutations in the spliceosome machinery (most notably SF3B1) are quite common in MDS but much rarer in AML (except for U2AF1). Finally, while the prevalence of karyotypic abnormalities is fairly similar between AML and MDS (40% vs. 50%), the chromosomal fusions, inversions and translocations are distinct for each disease (Hiddemann et al., 1986).

5. Gene and hematopoietic stem cell therapies

The hematopoietic system is likely the system where both stem cell therapy and gene therapy are easiest to achieve. Having said that, it of course not an entirely trivial matter. Given that most mature blood cells have a relatively short lifespan and all arise from the undifferentiated HSPCs, genetically modifying the HSPCs will subsequently propagate the corrected gene into the mature effector cells, alleviating the disease phenotype. This is in stark contrast to genetic disorders affecting mature solid-state organs; it is much harder to do gene therapy on the brain or the kidneys for instance. Several serious diseases of the blood and immune system have been cured by gene therapy of HSPCs, including X-linked severe combined immunodeficiency (X-SCID) (De Ravin et al., 2016), chronic granulomatous disease (CGD) (Grez et al., 2011), Wiskott-Aldrich Syndrome (WAS) (Aiuti et al., 2013) and Adrenoleukodystrophy (Cartier et al., 2009).

The terms hematopoietic stem cell transplantation (HSCT) and bone marrow transplantation (BMT) are sometimes used interchangeably. Since actual HSCT is never done (transplantation of mature progenitors is important to shorten the period of immunosuppression) and is practically impossible currently (we cannot isolate HSCs to that level of purity), I will use the more correct term “BMT” throughout this thesis.

5.1. History

Research into BMT began over a decade before the hallmark experiments of Till and McCullough. In the wake of World War II, it was discovered that people exposed to the smallest lethal irradiation dose died of bone marrow failure. Spurred on by this, in 1949, Jacobson et al. showed that a mouse can survive lethal irradiation as long as the spleen is protected from the radiation (Jacobson, 1949). Following this, Lorenz and colleagues showed that splenic protection was unnecessary for survival, simple intravascular infusion of spleen or bone marrow cells was just as effective at saving the mice (Lorenz, 1951).

Following these discoveries, studies diversified into successfully treating leukemic mice (Barnes, 1956), and at almost the same time, treating human patients (Thomas, 1957). The human studies were however much less successful and only transient graft was observed, with subsequent bone marrow failure. The only condition that allowed long term engraftment in humans was when using an identical twin donor (Thomas, 1959). A review concluded that from over 200 allogeneic marrow transplants done in humans in the late 50s and early 60s, not a single one had been successful (Bortin, 1970). The critical part that was missing was the immunological matching, and after the discovery and clarification of the human leukocyte antigen (HLA) groups (Dausset, 1958; Miescher, 1954; van Rood, 1958), bone marrow transplantation in humans started experiencing the success we see today, with the several decades-long complication-free survival (Bach, 1968; Bortin, 1994; deKoning, 1969), and a new era in transplantation medicine was born.

5.2. Sources of human HSCs

Human HSPCs can be collected through needle aspiration of the bone marrow, usually the iliac crest or the sternum is punctured, or by simple venous blood collection after treating the donor with an HSPCs mobilizing agent such as plerixafor or cyclophosphamide. Umbilical cord blood was discovered in 1974 by Knudtzon and colleagues who showed that this blood, previously considered a complete useless by-product by everyone, contained cells showing HSPC activity, at least *in vitro* (Knudtzon, 1974) (Figure 5). However, this did not generate a lot of interest before it was shown that these cells were capable of multilineage reconstitution *in vivo* and could be handled, stored and frozen without any detrimental effects to their activity (Broxmeyer et al., 1989) (Koike, 1983). After the first cord blood transplantation was successfully performed in 1988, curing a child with Fanconi anemia (Gluckman et al., 1989), considerable efforts have been

made to improve and broaden the applicability of this therapy, being able to offer it to a wide clientele of patients.

A critical advantage of CB-HSCs is their "immunological immaturity", in effect not requiring the same rigorous matching between donor and patient as from adult sources (Petersdorf et al., 1998), possibly due to the presence of fewer activated/mature lymphocytes, particularly T-cells, the main cause of graft-vs-host disease (GvHD) (Garderet et al., 1998). However, the disadvantage is the limited amount of engraftable HSCs and prolonged neutropenia. These problems, particularly the first one, have dominated the field since its inception but we are finally starting to solve the first one. Many positive regulators of CB-derived HSPCs-expansion have been identified, and several have made it to clinical trials showing quite encouraging preliminary results.

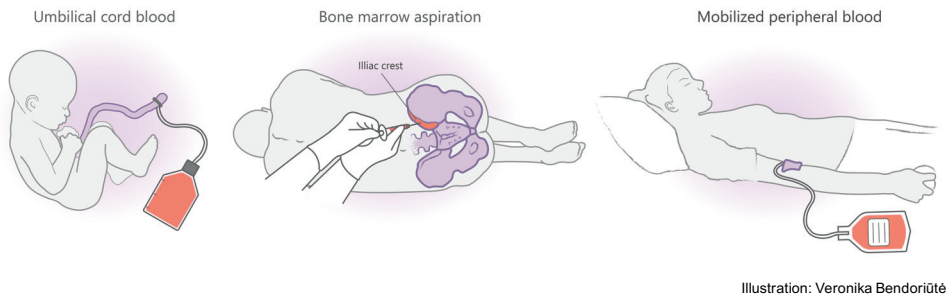

Figure 5. The three different sources of HSCs for bone marrow transplantation as well as research.

5.3. Bone marrow transplantation

BMT can be performed for several reasons. Possibly the most common one is in treating leukemias and lymphomas. In this case, a suitable donor has to be found, preferably with four or five matches of the six major histocompatibility complex (MHC) proteins. Since there are over 1000 different variants of each, this is sometimes quite challenging. When possible, a close relative (sibling, parent or child) could be the donor. In the context of cancer, it has been shown that it is even advantageous not to have complete six out of six match, since this triggers a graft vs. leukemia (GvL) effect, killing off the remaining malignant cells, and the leukemic stem cells (LSCs) in particular that are resistant to the chemotherapy used prior to BMT.

The second reason is to safeguard cells from high dose irradiation or chemotherapy. Here, the patient's own BM is harvested and stored. After

collection, the patient is exposed to high dose chemotherapy or irradiation in an attempt to cure a malignancy. Since enough HSPCs to repopulate an adult are kept outside the body, the patient can be treated with significantly higher doses of cytotoxic agents, doses that otherwise would have killed the blood system. Multiple myeloma (MM) for instance, can be treated in this manner. Naturally, this can only be done for cancers that can be cured with high dose chemotherapy and that with great certainty have not engaged the bone marrow; there is currently no possible way to discern an LSC from an HSC, and however tempting it might be to harvest the bone marrow of a leukemia patients, in the absence of a donor for instance, this strategy is pointless.

Third, BMT can also be used as an immunological "reset", engaging other medical specializations apart from hematology. This is usually used to treat highly aggressive autoimmune diseases such as primary progressive multiple sclerosis (MS) or disease modifying anti-rheumatic drug (DMARD) resistant rheumatoid arthritis (RA). The harvesting and chemotherapeutic procedure is almost identical as in the second case, however here the chemotherapy is actually given with the purpose of destroying the hematopoietic and immune systems. When the collected HSPCs are re-infused, no disease maintaining lymphocytes are left, and since there are no lymphocytic memory cells, the risks of relapse are quite small.

And finally, BMT is used to correct a genetic disease in the hematopoietic system. Usually, no conditioning is required, cells are merely harvested, the erroneous gene is corrected *in vitro*, and the genetically modified cells are reintroduced back into the patients. For many diseases, a complete (100%) function of a particular gene is rarely required, often mere 10-25% expression is enough. This number is much easier to achieve with the safer gene therapy delivery vectors.

5.4. Improvements and future challenges for BMT

Despite BMT having been performed for many decades, there several areas where major improvements are desired (Figure 6). First, there is a requirement of safe gene delivery systems. For gene therapy within the hematopoietic system, lentiviruses are preferentially used, and while their safety profile in terms of insertional mutagenesis and the ability to infect non-dividing cells makes them more desirable than gammaretroviral vectors for instance, there is, at least currently, always a hazard when using viral vectors. A small clinical pilot study attempting to correct the mutation causing WAS, reported seven cases of leukemia out of the thirteen boys enrolled in the clinical trial (Braun et al., 2014). Since there have been major improvements in the treatment of pediatric malignancies, and six of the seven children were cured. However, this example clearly illustrates the need for safer vectors.

Second, and perhaps even more important, is the dire need of safer conditioning regimens. A common cause of death in people over the age of 65 with hematological malignancies is their frailty, making it impossible for them to survive the conditioning regimens prior to BMT. There have been several studies in the murine system, showing successful engraftment of donor marrow cells without radiation or chemotherapy, using a CD45-immunotoxin for instance, however these are still in the pre-clinical stage (Palchaudhuri et al., 2016).

Third is the lack of matched donors. A significant group of patients in need cannot receive BMT therapy due to the lack of a suitable donor. For some minorities, the situation is even more difficult. Cord blood is one option, allowing less rigorous MHC matching, but cells from this source usually too few to engraft an adult and due to their immature nature, cause approximately two-three-fold longer period of immunosuppression, significantly limiting the use of cord blood. Clearly, development of safe and efficient ways of expanding engraftable human cord blood-derived HSCs is of utmost importance. Several strategies can be used to address this, for instance; engineering a matching donor sample through gene therapy on donor MHC loci, developing more effective ways of expanding and differentiating HSPCs derived from CB, or generating patient-specific HSCs from induced pluripotent stem cells (iPSC). Regardless, improvements in any of these areas are likely to benefit millions of patients annually.

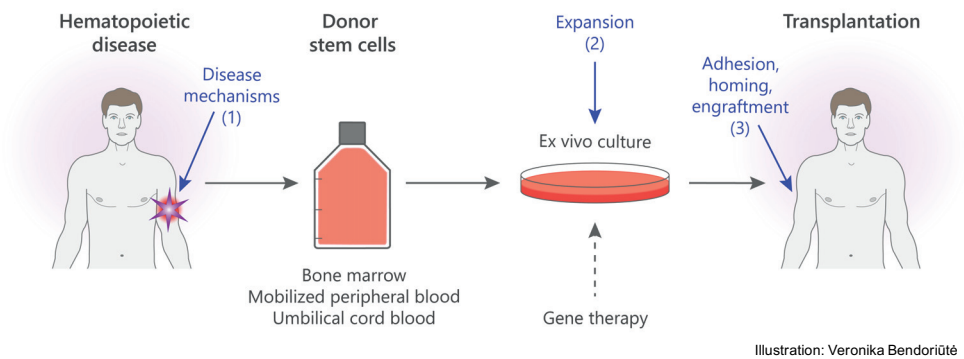

Figure 6. The three points of attack to improve treatment of hematological malignancies. Developing safer conditioning regimens is not illustrated here.

6. Targeted therapies for hematopoietic malignancies

Possibly, the best example of the success of targeted therapies is the treatment and cure of acute promyelocytic leukemia (APL), a subtype of AML. In 95% of this disease, there is a translocation involving the retinoic acid receptor-alpha (RARA) on chromosome 17 with the promyelocytic leukemia gene (PML) on chromosome 15, abbreviated t(15;17). This fusion creates a hybrid protein that blocks transcription and differentiation of granulocytes. Since the fusion protein involves the RARA gene, APL responds quite well to Vitamin A therapy; treating patients with vitamin A derivates lifts the differentiation block imposed on the granulocytic progenitors by the PML-RARA fusion gene, allowing terminal differentiation. The remission induced by Vitamin A is often short-lived and ordinary chemotherapy (primarily anthracyclines) is required as well as the antibody-drug conjugate gemtuzumab-ozogamicin that delivers toxic substances to the myeloid CD33-positive cells. Nonetheless, the combination of these two targeted therapies gives APL the best overall prognosis and survival compared to other AML subtypes, with no requirement for BMT or high dose chemotherapy consolidation.

Curing other subtypes of AML and other leukemias is a challenge, even when using targeted therapies. Similar to APL, CML is caused by a translocation between the BCR gene, a serine/threonine kinase (STK) without a completely clear function, on chromosome 22 and ABL1, another cytoplastic and nuclear STK located on chromosome 9. Upon the t(9;22) translocation in HSCs, this creates the BCR-ABL fusion protein that pushes the HSCs into overdrive, producing more and more mature blood cells, particularly leukocytes. Interestingly, this is achieved without any overt HSC exhaustion. Primary treatment (Dubash et al., 2013) is tyrosine kinase inhibitors (TKIs) such as imatinib, dasatinib and ponatinib. While few patients get cured with this therapy, the majority have to stay on this drug for life, and in an unlucky few, the leukemia becomes resistant, causing increased blast levels and eventually transformation to AML.

Over 95% of MPNs are caused by mutations in either MPL, JAK2 or CALR, where over 50% are driven by JAK2. Because of these few, clearly defined mutations, MPNs are also amenable to targeted therapy, and several inhibitors of mutated JAK2 have been developed including ruxolitinib, lestaurtinib and pacritinib, all having a similar therapeutic principle as the BCR-ABL inhibitors for CML.

A critical difference between these examples and AML and MDS is the complexity, while CML and MPNs are caused by the same mutations in only a few genes for most patients, AML and MDS are genetically much more diverse. It is therefore a larger challenge to offer targeted therapies for patients with the latter two diseases. Furthermore, even the TKIs have many off-target effects; sorafenib for instance, is an antagonist of vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR) and RAF-kinases in addition to FLT3. Nonetheless, given the successes of targeted therapies in hematopoietic malignancies, including AML with specific mutations, and our assumption that not all causative mutations have been identified to date, I explored the possibility of identifying previously unknown driver mutations in AML and MDS (**paper IV**). The road from the discovery to development of a corrective therapy for that particular gene is quite long, but it is my hope that with added unraveling of the complexity of this disease, we might come closer to offering better therapies for AML and MDS patients.

RNA INTERFERENCE

1. RNA interference – Basic principles and discovery

A critical element of posttranscriptional regulation is RNA-interference. Conceptually, it a process where nascent mRNA that has been transcribed in the nucleus and exported to the cytosol for translation, before its loading onto the ribosome, is bound by another RNA molecule (the interfering one), and by doing that, instead of resulting in translation, leads to degradation of the mRNA. This seemingly "ineffective" process has likely evolved in order to finetune transcription control to meet specific functional requirements, quite possibly enabling the development and evolution of more complex, multicellular organisms. While the response at the post-transcriptional level is not as rapid as on the post-translational, this also likely allows a cell to respond quicker to changes in the environment.

The discovery of RNA-interference (RNAi) was awarded the Nobel Prize in Physiology or Medicine in 2006 to Andrew Z. Fire and Craig Mello for the nominal work published in Nature in 1998 (Fire et al., 1998). Here, the authors were trying to understand how gene expression is regulated in the nematode worm *Caenorhabditis elegans* (*C. elegans*), a very common model organism in molecular biological research. RNAi is a critical gene regulatory mechanism that is deeply evolutionary conserved; from yeast to simple plants to worms to humans (Nellen and Lichtenstein, 1993). Additionally, the authors showed that this process was catalytic where only a tiny amount of interfering RNA was required to achieve silencing and how this process can spread between cells, which previously was thought not to happen due to the size and charge of the RNA molecule. This paper thus heralded an entire new research area in molecular biology.

2. RNAi – from Saccharomyces to Homo sapiens

2.1. Exogenous and endogenous RNAi

RNA interference adds a complex and versatile layer of gene expression control at the post-transcriptional level. RNAi can be divided into two broad categories: microRNA (miRNA) and short interfering RNA (siRNA), where short hairpin

RNA (shRNA) is a subtype of the latter and the RNA type that has been used throughout this thesis (Carthew and Sontheimer, 2009).

There are two main differences between miRNA and siRNA. miRNAs are endogenously expressed from the cells own genome. In humans, it is hypothethized that over 5% of the genome is encoding over 1000 miRNAs that fine-tune and regulate the expression of over 30% of all genes (Jinek and Doudna, 2009; Macfarlane and Murphy, 2010). Given that miRNAs control such a large fraction of known human genes, they have been implicated in a wide and diverse range of cellular processes; ranging from cell growth and tissue differentiation to formation of hetero- and euchromatin. Concordantly, dysfunction of miRNAs have been implicated in a wide range of diseases including the cardiovascular system, central nervous system, as well as cancer (Lu et al., 2008). A quite interesting observation is that while the size of the genome does not correlate with the complexity or the intelligence of an organism in any way (the rare Japanese flower *Paris Japonica* has around 150 billion bases (Pellicer et al., 2010), over 45 times more than humans) the number of miRNA does seem to do so, where humans have the highest number known to date (Berezikov, 2011).

The term "siRNA" on the other hand, while it can be endogenously derived as well, is typically used to describe either exogenous synthethic RNAi that has been delivered into a cell using electroporation, liposomal particles or a virus. The cellular response to a true viral infection also induces the siRNA machinery, in this case endogenously (Carthew and Sontheimer, 2009). The final product of both these pathways is very similar; an Argonaute protein bound to a single stranded RNA (ssRNA) ~20-30nt long, that binds to mRNA with full or near-full complementarity and thus induces either degradation of the mRNA or repressed transcription.

RNAi achieves gene level reduction through several mechanisms; binding of the complementary strand can lead to cleavage, physical blocking leads to direct translation repression or induction of deadenylation with subsequent mRNA destabilization and decay (Jackson and Standart, 2007). siRNAs usually feature perfect complementarity with the target strand, while miRNAs contain mismatches. Presumably, this reflects their individual biological role; siRNAs are synthetized as a direct response to a particular process or infection while miRNAs function as fine-tuning molecules. Mechanistically, this causes various degrees of silencing in proportion to the siRNAs and miRNAs complementarity to the target sequence. Given their profound role in pathology, RNAi has been exploited for therapeutic purposes with considerable success, with several projects now in clinical trials (Lam et al., 2015).

2.2. The mechanism of RNA interference

Whether the RNAi molecule is endogenously expressed or exogenously introduced into the hosts genome, it follows the same sequence of transcription and processing (Figure 7). The genesis of the si/miRNA begins in the nucleus where a primary transcript is produced, usually >1000 nt long, containing single or multiple double stranded si/miRNA molecules (Saini et al., 2007). There is conflicting data whether RNA polymerase II or RNA polymerase III is responsible for transcription of miRNAs (Borchert et al., 2006; Lee et al., 2004). However, given the critical importance of this post-transcriptional gene regulatory mechanism, it is likely that a level of redundancy is involved. Exogenously introduced siRNA can also be transcribed either by Pol II or Pol III, depending upon which promoter is designed to drive the expression of the exogenous construct. In this thesis, the common RNA polymerase III U6 promoter in the pLKO.1-vector has been used.

After transcription, the pre-si/miRNA is cleaved by the microprocessor complex comprising the RNase III family enzyme Drosha, and the dsRNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8) (Kim and Kim, 2007). This results in a ~65-70nt long precursor si/miRNA (pre-si/miRNA) that is exported to the cytoplasm by Exportin 5 and RanGTP (Lund and Dahlberg, 2006). Depending on the length of the pre-si/miRNA, different numbers of pre-si/miRNA are produced.

In the cytoplasm, the pre-si/miRNA is further trimmed by the Dicer enzyme (a protein that also contains RNase III domains, like Drosha), resulting in a 21-25 nt long dsRNA (Schwarz et al., 2003; Shabalina and Koonin, 2008). The cleaved dsRNA, while being bound to Dicer, is simultaneously bound by an additional dsRNA-binding protein (dsRBP), and subsequently loaded onto an Argonaute family protein. Upon loading of the pre-si/miRNA to Argonaute, Dicer and the dsRBP dissociate, and the formed ribonucleoprotein complex of the pre-si/miRNA with Argonaute is called the RNA-induced silencing complex (RISC). Dicer and dsRBP, while being pivotal in bringing pre-si/miRNA and Argonaute together, are not required to mediate the RNA-interference, they comprise the RISC-loading complex (RLC) but are not part of RISC themselves (MacRae et al., 2008). In human cells, the effectiveness in terms of expression and target gene silencing of the long dsRNA rapidly leads to interferon response and apoptosis, and is therefore an unsuitable construct. Therefore, the optimal range, yielding the highest possible specificity while avoiding the interferon response, is around 21-23 nucleotides (Elbashir et al., 2001).

The least understood process of the RNAi pathway is strand selection, which happens after loading of the dsRNA onto RISC. For RNA mediated silencing to

occur, one strand needs to dissociate from RISC exposing the hydrogen bonds of the other strand to base pair with endogenous mRNAs present in the cytosol. While it is known that the exonuclease C3PO is critical to ensure degradation of the passenger strand, much less is known about how C3PO "knows" which strand to degrade (Hu et al., 2009; Ye et al., 2011).

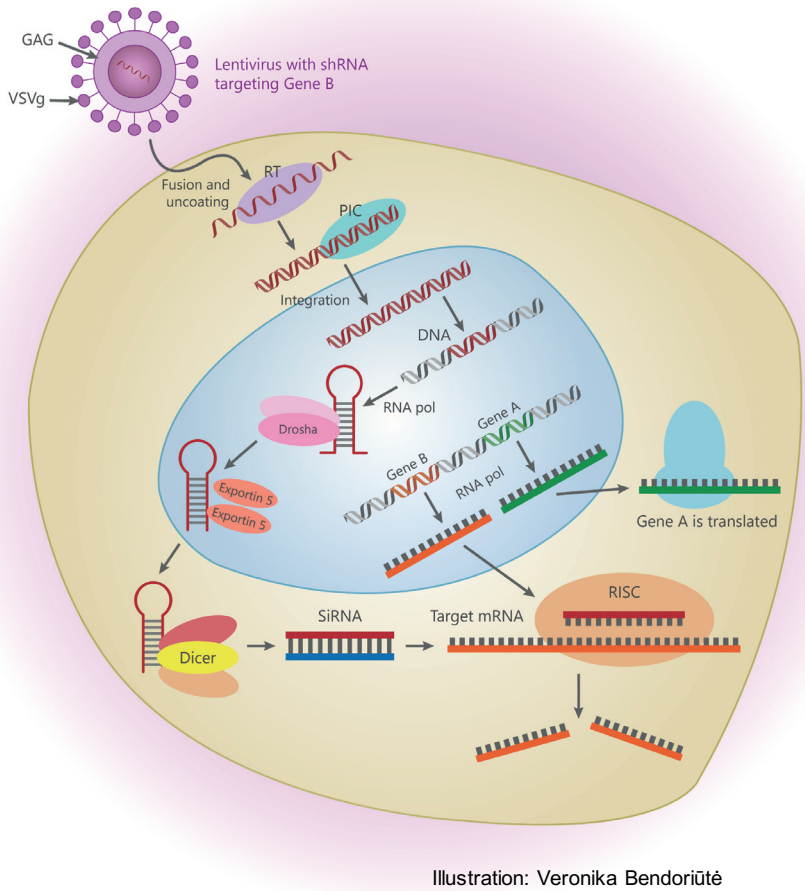

Figure 7. Schematic illustration of RNA interference. PIC: pre-integration complex, RT: reverse transcriptase, RISC: RNA-induced silencing complex.

After passenger strand degradation, the active RISC (containing a single-stranded RNA (ssRNA)), is free to move around the cytosol binding to mRNAs that show sequence complementarity. The complementarity need not be perfect; rather, the first 2-6 nucleotides are the pivotal seed sequences, initializing binding of RISC to the mRNA but the subsequent nucleotides can have several mismatches. This is a

key reason to the unspecific, or "off-target" effects of RNAi as will be described below (Wilson and Doudna, 2013).

In the case of perfect complementarity, the target mRNA can be cleaved immediately if the Argonaute protein bound to the guide ssRNA has a catalytic domain. Interestingly, out of the four different Argonaute proteins found in human cells (AGO1-4), only AGO2 has this property (Janowski et al., 2006). However, even in the context of incomplete match, RISC can induce transcriptional repression by other, non-endonucleolytic, mechanisms such deanylation and destabilization of the mRNA and ribosome stalling (Fabian et al., 2009). Regardless of the mechanism, the level of the translated product decreases, where the magnitude of the decrease is multi-factorial involving base-pair complementarity, the Argonaute isoform that the siRNA is bound to, and the presence of other mRNAs with sequence similarities acting as a "sponge" for the siRNA, in effect, diluting out the specific gene knockdown (Wilson and Doudna, 2013).

3. Using RNAi to study gene function

3.1. RNA interference in the study of hematopoiesis

Probably, the most well-established approach to identify genetic regulators is using reverse genetics. In reverse genetics, the correlation of "gene to phenotype" is used, where a gene is disrupted or overexpressed and the phenotype is subsequently scored and evaluated. Conversely, *forward genetics* starts with a certain (pre)defined phenotype, and then, the genes underlying this phenotype are identified.

An early and crude example of forward genetics in understanding gene function was through unspecific mutagenesis, the so-called Ames test; bacteria would be subjected to a mutation inducing chemical agent, and subsequently cultured on plates where they normally would be unable to grow; a plate containing an atypical growth nutrient or an antibiotic. Bacteria that managed to grow in these conditions would be isolated and the causative mutation identified (Ames et al., 1973). Naturally, this approach is quite laborious, particularly identifying the causative mutation, and does not lend itself as easy when studying eukaryotic cells, and human cells in particular.

Selecting candidate genes for the reverse genetics approach can often be done using transcriptome analysis and functional data from different studies and contexts. When studying the hematopoietic system, one option would be to sort

out different HSPC populations, including HSCs as in (Laurenti et al., 2013), and compare the expression profile of HSCs to more downstream progenitors. The hypothesis is that certain differentially expressed genes have significance for proper function of the HSCs. To investigate whether this is the case, the genes are disrupted one at a time and the subsequent phenotype and effects of this disruption are assayed and characterized. Indeed, the phenotypic validation is often combined with transcriptomic approaches as well, creating a process reminiscent of a positive feedback loop; transcriptome analyses give hints about novel regulatory genes of interest. The novel gene is then disrupted in the same manner as the previous ones and subjected to the same phenotype characterization and transcriptomic analysis. This characterization likely identifies additional candidate regulators among the differentially expressed genes, essentially restarting the process. Continuation in this manner eventually allows mapping of complex regulatory networks.

This classic strategy has three major drawbacks. First, it is quite time consuming particularly since complete knockout mouse models often have to be generated. Second, this strategy is mostly limited to intrinsic regulators of HSCs, although knockout in a cell that is part of the hematopoietic niche in the bone marrow could be done as well. Third, some prior knowledge is required, since this process is too cumbersome and costly to be done randomly.

A completely different concept is to instead use the forward genetics approach by performing screens. Screens are done under a few assumptions. First, by adding different chemicals or nucleic acids to different cells, a seemingly homogenous cell population is made heterogeneous. Second, a selection pressure has to be applied to the cultured cells; for instance, a requirement to maintain stem cell activity or simply proliferate. Cells whose modification gives an advantage under such conditions will increase in numbers, and this is assayed at the end time point.

In the simplest format, a screen would be done in an arrayed fashion (Figure 8) where cells are plated in individual wells and different chemicals, ORFs or siRNA/shRNA molecules are added. The effects of each compound are then assayed in terms of cell proliferation and or/stem cell maintenance (Moffat et al., 2006). Unless automated, this format limits the number of compounds that can be screened in this fashion. To circumvent the limitation of the systemic format, pooled screens can be used (Paddison et al., 2004; Westbrook et al., 2005). Here, cDNA, si/shRNA, transposons etc. are screened in parallel, and the desired phenotype (such as proliferation or stem cell maintenance) is read out in the end (Figure 9). The pooled nucleic acid screen becomes a very powerful tool when combined with NGS (Church, 2006; Mardis, 2008), where each sequence in effect also serves as a molecular barcode. Thus, NGS is used as a “transduce and track” method, where the changes in relative frequency of each shRNA before and after

culture is a direct representation of that particular hairpins effect of cell proliferation and/or stem cell activity. In the context of RNAi screening, NGS has made it possible to screen libraries containing hundreds of thousands of shRNAs using the pooled setup without increasing the workload in any significant way.

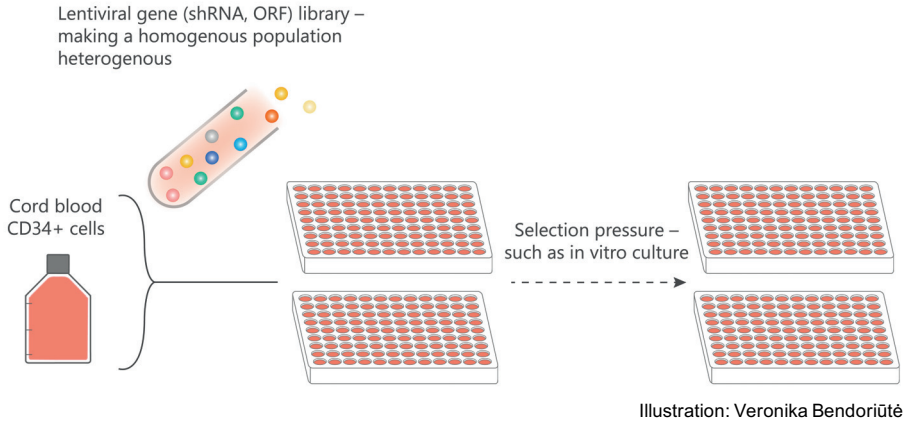

Figure 8. Outline of an arrayed screen.

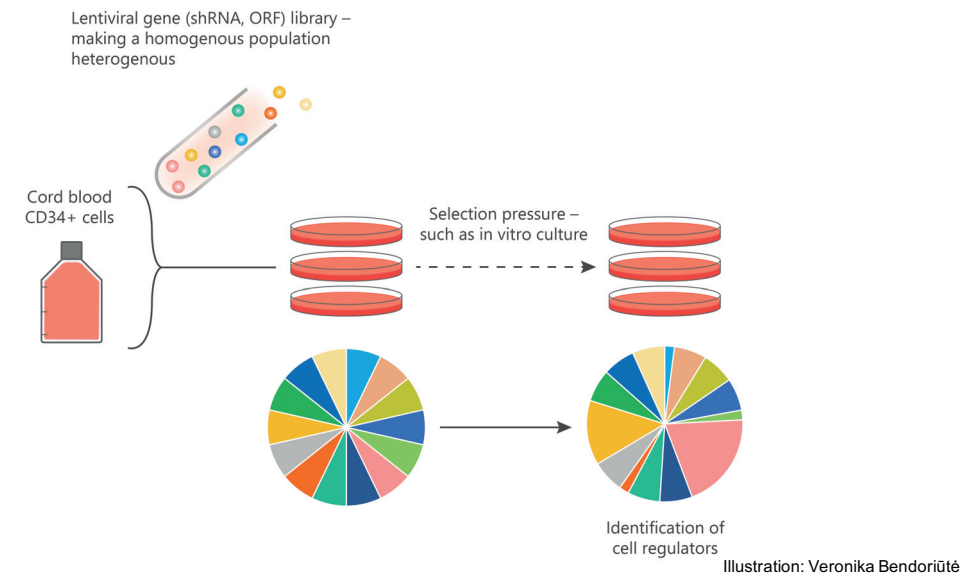

Figure 9. Outline of a pooled screen.

Achieving the same level of coverage for each sequence is not often possible in the pooled setting, where sub-optimal representation of the library to be screened easily causes sequences to be lost. Another drawback is of course that small molecular compounds (i.e. chemicals) cannot be screened in a pooled fashion. However, despite these drawbacks, the pooled screen is significantly more time and cost efficient (Karlsson et al., 2014). and are an attractive option where thousands of hairpins can be assayed at once, drastically decreasing the cost and time expenditure compared to the arrayed format. On the other hand, large pooled screens may not be feasible for certain cell types and assays, particularly when complex phenotypes need to be assayed visually, although automated imaging acquisition and analysis has improved drastically in recent years, making storage space more of a limiting factor (Karlsson et al., 2014).

After the discovery of RNAi in 1998 *C. elegans*, the concept of RNAi screening was initially introduced and used in that same organism (Tabara et al., 1998). *C. elegans* was found to be quite amenable to RNA interference, not only from a mechanistic but also from a practical approach when it was discovered that culturing this worm in the presence of bacteria engineered to effectively express double-stranded interfering RNA effectively led to ingestion of bacteria by *C. elegans* and subsequent induction of the RNAi machinery (Timmons et al., 2001). It was then relatively easy to apply the concept from classical genetics together with a molecular approach, identifying the RNA responsible for the phenotype. In *C. elegans*, it was almost equally easy to perform RNAi screens *in vivo* as *in vitro*.

Quite soon thereafter, the concept of RNAi was introduced in Drosophila, where RNAi screens were quite successful not least because of the low genetic redundancy in Drosophila and a high efficiency of RNA transfer, expression and silencing (Boutros and Ahringer, 2008; Clemens et al., 2000). However, *in vivo* screens were not as technically easy. Simple feeding of transgenic bacteria does not work in Drosophila, although RNAi could be injected into the developing embryo. To achieve *in vivo* screening in Drosophila, it is instead more feasible to generate a library of transgenic flies, each expression of dsRNA hairpin (>200 bp) that upon cross breeding become activated. Subjecting these flies with different dsRNA inserts to various selection pressure (nutrient depletion, temperature changes etc.) will allow identification of flies and sequences conferring that particular survival advantage (Dietzl et al., 2007). Needless to say, the generation of such “fly libraries” is extensively laborious, and a more feasible approach in Drosophila would be simpler *in vitro* screens after establishing cell lines originating from different tissues of the fly (Bai et al., 2008).

More recently, multiple commercial genome-wide shRNA libraries targeting several different mammalian cells have been generated, where the shRNA construct can either be packaged into lentiviral particles for transduction or simply

transfected into cells. Screens of this kind can be performed either individually or in pools. Since the creation of large lentiviral libraries (Figure 10 and 11), RNAi makes it now possible to perform pooled genetic screens, silencing thousands of genes in both cell lines and primary mammalian cells, including human (Campeau and Gobeil, 2011; Echeverri and Perrimon, 2006; Moffat and Sabatini, 2006).

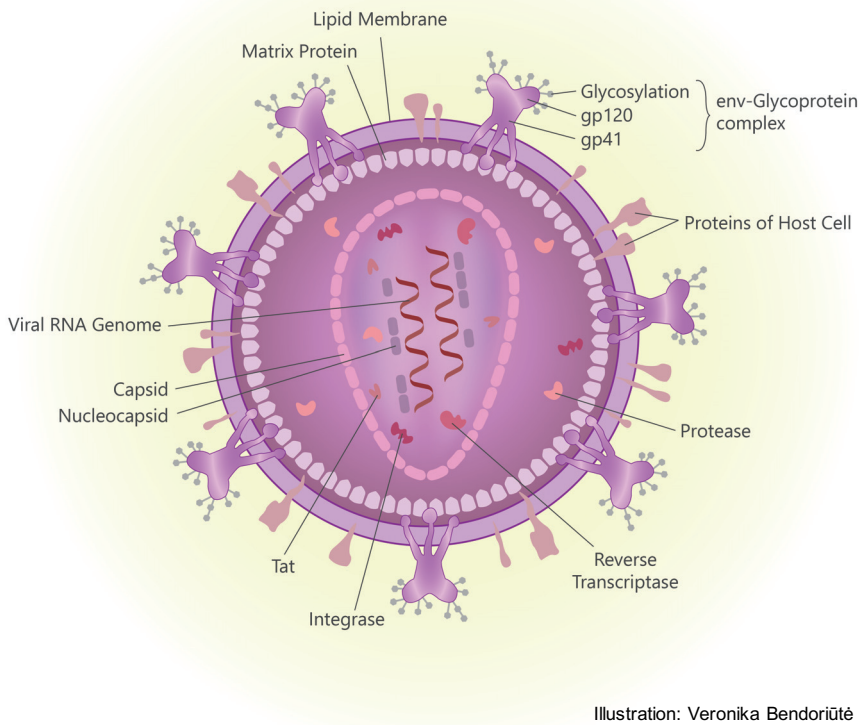

Illustration: Veronika Bendoriūtė

Figure 10. Structure of the HIV-based lentivirus, often used in gene therapy and to deliver RNAi vectors.

RNAi is fairly easily introduced into cells, virtually any gene can be targeted, and by keeping the complementary interfering sequence short, the negative effects are lowered. Furthermore, RNAi can be used in both reverse and forward genetics; a gene can be knocked down with subsequent classic phenotype characterization studies, but also, multiple RNA sequences can be designed, targeting different genes with different RNA introduced into different cells. Applying a selection pressure as in the Ames test would then constitute a forward genetics approach. In summary, one could say that RNAi screens are forward genetics screens that use molecular biology tools from reverse genetics.

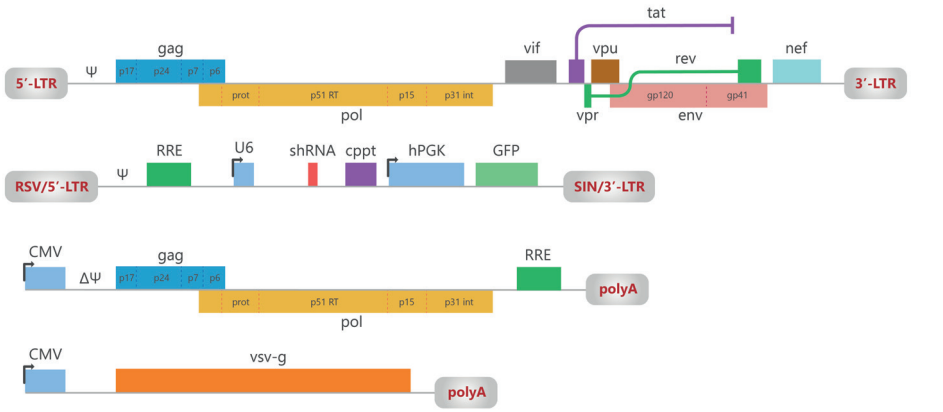

Figure 11. Genome of the native and engineered lentivirus. The top panel illustrates the nine genes constituting the genome of wild-type HIV. The three panels below illustrate the HIV virus redesigned to introduce specific genetic material into the host cells genome, in this case an shRNA construct. The shRNA insert, viral envelope and enzymes are all expressed from different plasmids. Since there are no packaging sequences on the plasmids coding for the viral envelope and enzymes (bottom two panels), they do not get inserted into the newly assembled virion, making the produced virus lack any replicative abilities.

3.2. Identification of HSC regulators using RNAi screens

Identifying regulators of HSCs has been a primary goal in hematology for a long time for several reasons; (i) to improve bone marrow transplantation (BMT), expanding HSCs *in vitro*, thereby being able to offer this therapy to a broader group of patients, (ii) to understand the changes that occur upon aging, establishment of clonal hematopoiesis and formation of malignancies, identifying novel therapeutic targets towards this, (iii) to get a better and deeper understanding of the blood cell development process from a biological and academic point of view.

The blood system renders itself particularly well to study through RNAi-screens. The success of RNAi screens depends in part on the ability of lentiviral RNAi-vectors to infect a sufficient number of target cells, ensuring an adequate coverage, as well as having the tools to design a readout assay with a high enough specificity and sensitivity (Galeev et al., 2017) (Figures 10 and 11). HSPCs have advantages in both respects, they can be acquired and transduced in sufficient quantities (Woods et al., 2000), are easily cultured, and flow cytometry can be used to sort out any desired phenotypic sub-population. Since HSPCs are only able to maintain their undifferentiated state for a short time in culture, this limitation can be used as a functional selection criterion where augmented expression of a certain cell

surface marker or simply increased proliferation can be detected by positive selection.

Using both the aforementioned genome-wide libraries as well as shorter, custom-made ones, many RNAi screens have been performed in both murine and human hematopoietic systems. In 2010, using a custom-made library targeting 20 known polarity factors, Hope et al. did a selected screen in murine HSCs, identifying Msi2 as a positive HSC regulator and Prox1 as a negative one (Hope et al., 2010). In 2011, screening a custom-made library targeting chromatin regulators in AML cells *in vivo*, Zuber et al. identified Brd4 as a critical and selective negative regulator of leukemic stem cells, where knockdown of Brd4 had a strong antiproliferative effect on leukemic cells but only a very mild phenotype on wild-type healthy cells (Zuber et al., 2011).

DNA-damage response has always been a critical issue not just in HSCs but also in stem cell biology in general. Screening 947 cancer related genes in an *in vivo* RNAi screen (transplanting transduced HSPCs and analyzing the relative shRNA distribution weeks after engraftment) identified Batf as a mediator of cell cycle arrest upon DNA damage in murine HSPCs (Wang et al., 2012). Continuing on the interesting topic of *in vivo* screens, Miller et al. identified the exclusive dependence on ITGB3 signaling of leukemic cells, something that was not required for healthy human HSPCs (Miller et al., 2013). Naturally, the findings of Zuber and Miller make Brd4 and ITGB3 very attractive novel therapeutic targets for AML. Building on the knowledge of AML development, an elegant *in vivo* screen performed by Puram et al. identified circadian rhythm transcription factors Clock and Bmal1 as genes crucial for growth of leukemic cells *in vitro* and *in vivo*. Depleting these genes produced anti-leukemic effects while sparing the healthy HSCs, opening an additional therapeutic opportunity (Puram et al., 2016). In our lab, we have successfully used RNAi screening to identify regulators of cell adhesion (Rak et al., 2017), a process critical to increase engraftment efficiency after BMT, as well as novel regulators of self-renewal (Baudet et al., 2012; Galeev et al., 2016; Kinkel et al., 2015), where the latter three are presented as articles in this thesis.

More recently, the discovery and development of clustered regularly interspaced short palindromic repeats (CRISPR) technology has become a powerful tool to not only, more selectively attenuate or knockout genes with high precision, but also overexpress genes to reasonable levels and not the supraphysiological levels achieved by classic open reading frame (ORF) overexpression vectors (Cong et al., 2013; Shalem et al., 2014; Wang et al., 2014). Two key advantages of CRISPR over RNAi are the significantly lower off-target effects and the ability to modify any region in the genome such as enhancers or promoters; since the modification occurs on the DNA in contrast to the RNA level, no expression is required. While

this technology works quite well in cell lines, using the CRISPR system to modify gene expression in primary cells, especially human, has been harder, although significant improvements have been made using human $CD34^+$ as well as other primary cells (Tothova et al., 2017). Much work therefore remains to be done, but once achieved it is doubtless that CRISPR together with RNAi will be a very strong combination for studying gene function.

AIMS OF THIS THESIS

In this thesis, I have focused on identifying novel intrinsic regulators of human HSPCs, with the aim of (I) understanding the regulation of human CB-derived HSCs; which genes push the HSCs towards a particular cell fate, and attempting to differ between healthy, physiological expansion and that of leukemia. (II) Delineating the mechanisms that result in malignant hematopoiesis, specifically AML and MDS, with the aim of designing better, and more targeted therapies in the future.

With this goal in mind, I have tried to identify genetic regulators in the least-biased way possible, using the largest available RNAi library at the time to employ a near-genome wide approach, without any preconceptions about as to which genes are likely to be important and which are not, with no apparent grounding in previous studies, other than those showing the feasibility of this particular approach.

In is my hope that, with the findings presented in this thesis, we have understood the regulation of the human HSC a bit more, came a small step closer to improving BMT therapies, enabling us to offer it to more people, especially the elderly, but also helped us to identify novel therapeutic targets for myeloid malignancies, AML and MDS in particular.

SUMMARY OF ARTICLES

Paper I – RNAi screen identifies MAPK14 as a druggable suppressor of human hematopoietic stem cell expansion

In this paper, we focused specifically on identifying target genes that act as negative regulators of human HSC expansion *in vitro* and at the same time were druggable and could be targeted by specific molecular inhibitors. To this end, we used a pooled subset of the RNAi consortium (TRC), targeting around 1000 kinases and phosphatases with an average of 5 hairpins per gene. $CD34^+$ cells were put in culture and transduced with the aforementioned lentiviral library. Normally, the expression of CD34 on the cell surface is lost fairly rapidly as cells differentiate *in vitro*. Therefore, using the limited persistence of CD34 expression during culture as a functional selection tool, we isolated cells that still maintained CD34 expression after three weeks of *in vitro* culture. By directly comparing the relative distribution of each shRNA before and after the culture period, we ranked each individual shRNA based on its ability to expand or maintain CD34 expression.

To minimize noise of the assay, (more on this in "General discussion" below), we introduced stringent selection criteria; enrichment of multiple shRNAs per gene, validated (>50%) knockdown of each shRNA, expression of the gene of interest in $CD34^+$ cells and finally, whether the candidate gene could be targeted by a small molecule inhibitor. Using these criteria to rank all included genes, the highest scoring gene was mitogen-activated protein kinase 14 (MAPK14), having three individual hairpins in the enriched fraction. Using four different chemical inhibitors, we showed that attenuation of MAPK14 results in an increase of CD34-expressing HSPCs during a 5-week period of *in vitro* culture and cells treated with the MAPK14 inhibitors show higher chimerism levels upon transplantation *in vivo*. This study clearly illustrates the feasibility of using forward RNAi screens to identify regulators of hematopoietic stem cell activity, and was the basis for our future screens. Having shown the proof-of-concept and feasibility of our approach in this study, we scaled up our screening paradigm to do a near genome-wide screen (Paper II).

Paper II – Genome-wide RNAi screen identifies Cohesin genes as modifiers of renewal and differentiation in human HSCs

In paper II, due to our success in identifying a novel regulator of HSPCs in our previous study, we decided to widen our approach using a near genome-wide library to include 15 000 genes with an average of 5 hairpins per gene, yielding in effect, 75 000 shRNAs, using the same screening paradigm as in paper I. Given the vast depth of the data, we decided to focus not just on individual genes, but also include groups of genes belonging to the same gene family, cell signaling pathway or protein complex in an attempt to understand more complex aspects of gene regulation in human HSPCs. To this end, we ranked each "group of genes" based on the ability of each constituent gene to expand $CD34^+$ cells. Interestingly, we found that four out of five members of the Cohesin complex (STAG2, SMC3, RAD21 and STAG1), all scored within the top 1% of our gene list. Subsequent validation studies revealed that knockdown of each Cohesin component individually resulted in an increase of undifferentiated cells *in vitro* and *in vivo*, coupled with a myeloid skewing *in vivo* and the ability to engraft secondary recipients, resembling the early stages of a myeloid disorder where a significant fraction of engrafted cells showed atypical nuclei. Further, we showed that knockdown of any Cohesin component results in relative upregulation of HSC-specific genes. Whether this is a direct effect of Cohesin deficiency or just a consequence pertaining to the failure to upregulate differentiation promoting genes remains to be determined. Further, it is not quite clear whether the effect is direct or indirect, i.e. if HSC genes actually are upregulated or whether this is simply a consequence of performing the analysis in bulk, a mere consequence of increased HSC numbers.

Paper III – Jarid2 regulates hematopoietic stem cell function by acting with polycomb repressive complex 2

This work has been done in collaboration with Dr. Marnie E. Blewitts group in Melbourne, Australia. In the early phases of the analysis of our genome-wide screening data, we discovered that JARID2 was the highest-ranking DNA binding protein overall. Upon validation of this finding, we saw that knockdown of JARID2 resulted in expansion of HSPCs from multiple different sources; human

fetal liver, cord blood, as well as bone marrow, all showed increase in $CD34^+$ *in vitro*. Similarly, *in vivo*, knockdown of JARID2 resulted in increased human chimerism levels in blood and bone marrow of recipient animals, together with the ability to engraft secondary hosts and upregulation of HSC-specific genes. At the same time, our collaborators in Australia had done a similar discovery for Jarid2 in murine cells, derived both from fetal liver and adult bone marrow. Jarid2 appears to regulate HSPCs function by direction interaction with PRC2, recruiting this protein complex to the genome. The roles of PRC1 and PRC2 in gene regulation are very complex, but it has been shown before that at least in murine HSCs they appear to have antagonistic roles where PRC1 increases HSC-function while PRC2 decreases it. It is therefore a plausible mechanistic explanation that in the absence of Jarid2, PRC2 does not bind as effectively shifting the epigenetic landscape towards that of PRC1, thereby increasing HSC function.

Paper IV – Identification of potential disease associated genes in acute myeloid leukemia and myelodysplastic syndrome using RNAi screens and targeted sequencing

The nature of the genome-wide screen we conducted was focused on cell proliferation, an enrichment of tumor suppressor genes is therefore an expected finding. Indeed, JARID2 and Cohesin have been shown in many studies to be mutated in AML and MDS. Assuming that not all causative mutations in these two heterogeneous diseases are known, we reasoned that perhaps our screen preselects both known and unknown genes with tumor suppressor gene (TSG) function.

To investigate this, we designed custom DNA probes to target the top 500 genes from our genome-wide lentiviral shRNA screen and used these probes to sequence near 400 patients suffering from either AML or MDS. We then decided to focus on genes showing recurrent (≥2 patients), and deleterious (i.e. frame-shift, stop-gain/loss and splice site mutations) mutations in our data set. Using this analysis, we narrowed down the potential candidates from 500 to 17. The 17 candidate genes were functionally validated in our *in vitro* assay for proliferation and maintenance of immature markers as well as assaying knockdown efficiency and overall expression of the 17 candidate genes in hematopoietic cells. This further reduced the candidate number to two; non-SMC Condensin I complex subunit D2 (NCAPD2) and serum deprivation response (SDPR).

To investigate whether Ncapd2 and Sdpr are actively involved in AML or MDS pathogenesis, we decided to investigate the effects of their knockdown on the pre-leukemic FLT3-ITD mouse model background. Knockdown of Ncapd2 or Sdpr in

LSK cells from FLT3-ITD homozygous mice showed a strongly increased serial replating activity, although the effect on immature cell surface markers was moderate and there was no correlation between knockdown efficiency and phenotype, possibly due to lower levels of expression of Ncapd2 and Sdpr in the murine cells. We are currently planning on using other preleukemic mouse models and also fine tuning our transduction protocols and cell usage, in order to more closely mimic the natural leukemogenic process in humans. We hope this will help clarify and validate whether our two potential regulators indeed are involved in leukemogenesis.

Additionally, since AML and MDS are genetically heterogeneous, we are aiming to achieve a higher mutation resolution; sequencing additional patient samples; bone marrow aspirates taken upon diagnosis but also relapse, quantifying the disease burden in the bone marrow, all aiming to increase the specificity and sensitivity of our assay, where stringent preselection would likely increase the chances of generating a true, positive hit.

CONCLUSIONS

Forward RNAi screens are a successful strategy to identify regulators of cell fate of both normal and malignant hematopoiesis (papers I-IV).

As long as a feasible selection pressure and readout assay can be found, RNAi screens could likely be used on other cell types and for other purposes (papers I-III).

Inhibition of MAPK14 increases HSC-activity *in vitro* and *in vivo*, or at least prevents exhaustion (paper I).

JARID2, a PRC2 accessory factor regulates human and murine HSC-function through direct interaction with PRC2 (paper III).

Cohesin, a critical protein complex mediating proper sister-strand segregation during cell division and facilitating long-range chromatin interactions, is a critical regulator of the self-renewal and differentiation balance in human HSPCs (paper II).

Knockdown of Cohesin and JARID2 likely induces stem cell-specific gene expression programs in HSPCs (papers II, III).

NCAPD2 and SDPR are potential novel AML/MDS associated genes (paper IV).

Due to the limitations of the assay, there are likely more important HSC regulators that have yet to be validated (papers I-III).

Introducing stringent selection criteria when ranking shRNA and gene ranking decreases the noise of the assay substantially (paper I-IV).

Combining RNAi screens with NGS is a powerful tool to study regulators of cell fate in this context (papers I-IV).

GENERAL DISCUSSION

RNAi-based genetic screens

RNAi clearly has several limitations, causing both false negative and false positive hits. In the work presented here, false negative hits occur due to several reasons. First, due to different knockdown efficiencies of different shRNA molecules, a potentially important regulator could be missed due to insufficient knockdown. Second, the studies presented in this thesis have utilized cord blood derived $CD34^+$ HSPCs. This population is inherently heterogeneous where the actual engrafting stem cell frequency is quite low. The principle behind our RNAi screen is applying a selection pressure after making a homogeneous population heterogeneous; in this case, we transduce $CD34^+$ cells with a lentiviral library, where each cell has a unique shRNA giving them a certain advantage or disadvantage in *in vitro* culture. The application of a selection pressure, in this case simple *in vitro* culture since HSPCs tend to lose their CD34 expression under normal conditions, enables us to identify those shRNAs that have caused retainment or expansion of $CD34^+$ cells under these conditions.

The caveat is that $CD34^+$ are not particularly homogeneous, CD34 is also expressed on every upstream progenitor; CMP, GMP, MEP and CLP. Unfortunately, this expression is stable and no difference in expression levels are observed between an HSC and the different progenitor populations making it impossible to sort out cells based on cell surface expression levels. We are thus working with quite a heterogeneous population from the start, that after transduction becomes even more heterogeneous. Not every shRNA has then the opportunity to exert its proliferative effects; the results become a stochastic probability of which cell a particular shRNAs happens to hit.

There is, therefore, a substantial risk that the true HSC, or a more upstream progenitor at least, is not targeted, and a potentially important shRNA never "gets the chance" to exert its effect. Due to the fact that it is much harder to counteract the prevalence of false negative hits vs. false positive ones in this assay, there is a big chance that potential critical regulators were missed. Sadly, technical limitations make it infeasible to use a more defined, and thus more homogeneous population (for the work presented in this thesis, over 500 cords were collected).

The two main culprits behind false positive hits are insertional mutagenesis and off-target effects. The former happens when the strong promoter of the gene delivery vector happens to, by chance, get inserted next to a proto-oncogene,

causing extensive cell proliferation. While lentiviruses have a substantially lower risk of this event compared to older generation of viral vectors (such as the gamma retroviral vectors) (Cattoglio et al., 2007; Neschadim et al., 2007), it is not negligible and thus a point of concern. To counteract this phenomenon, multiple replicates are usually used, and we have used six transduction replicates, screening for candidates that perform well overall. It is an unlikely scenario for insertional mutagenesis to occur in the same locus in multiple replicates (even if certain regions are more insert prone than others). The latter; off-target effects, are significantly more difficult to compensate for in a screening setting. These are usually mediated by a fraction of the shRNA, usually 6-9 bases of the final 21-nucleotide RNA (Wilson and Doudna, 2013). Subsequent validation of presumptive hits is where off-target effects can truly be assayed, primarily by measuring knockdown efficiency; this is the strategy that was pursued in the studies described here. Naturally, separate transductions were carried out for the *in vitro* and *in vivo* studies as well. The *in vivo* model used here, the NSG mouse, has several limitations in its own right, discussed in chapter 3 in the first part of the thesis.

In paper IV, our aim was to identify novel regulators in AML and MDS. The 500 candidate genes that had the strongest expansion phenotype in the screen were sequenced in a cohort of near 400 patients. This rationale also poses the risk of generating false negative hits, since there are many more ways to silence or disrupt a gene than simply mutating its coding sequence. Epigenetic deregulation has become more and more appreciated in hematological disorders during the last decade, and disruption of the epigenetic landscape is believed to be an early event in leukemogenesis. While studies have shown that the epigenetic disruption is due to mutations on the genetic level of such regulators (Kotini et al., 2017), other mechanisms cannot be ruled out. Naturally, adding a complete epigenetic profile would have significantly improved on the study, however due to technical limitations, this was not possible to do to date.

Throughout the thesis, due to the ease of acquisition, human umbilical cord blood-derived HSPCs have been used for the majority of experiments. While many aspects of biology are quite similar between CB HSPCs and adult BM-derived HSPCs, there are still notably differences, not least in HSC activity and mutation load (as described in chapter 1 in the first part of the thesis). It is possible that a different gene list of negative regulators of proliferation would have been obtained had BM HSPCs been used for the screen instead. Also, for our leukemia study, using adult or even old human HSPCs could perhaps make it easier to trigger a leukemic transformation.

There is also the issue of bulk vs. single cell analysis, a topic that has received more and more attention in the last two-three years. Since each phenotypic

population within the hematopoietic hierarchy has been shown to be significantly more heterogeneous and less plastic than previously thought, it makes it difficult to ascertain the actual effects of our knockdown phenotypes, and a mechanism of action in particular. It is not clear whether an observed change in the transcriptome for instance, is due to actual changes in gene expression or simply a population shift. For practical reasons, not all assays have to be done on the single cell level, however, but to get a more thorough mechanistic picture, validation experiments, and gene expression/RNA-seq studies should. With the speed at which advances in molecular biology takes place, it is quite plausible that this will be widely available within a few years.

Cohesin as a novel regulator of HSPCs and a therapeutic target in leukemia

A major finding in this thesis has been the discovery of the cohesin complex as a novel regulator of HSPC fate. Cohesin is an evolutionary conserved protein, initially discovered already in 1985 (Larionov et al., 1985), where several subunits of this complex were identified as critical in maintaining proper chromosome segregation in *Saccharomyces cerevisiae* (*S. cerevisiae*) (Guacci et al., 1997; Michaelis et al., 1997). Several years later, the same function was attributed to the mammalian cohesin complex (Nasmyth and Haering, 2009), where cells lacking cohesin develop genome instability and aneuploidy (Remeseiro et al., 2012). Therefore, when the mutations in this complex were described as causing AML and MDS in 2012 (2013; Welch et al., 2012), it was initially believed that this was due to genomic instability caused by deficient cohesin, improper chromosome segregation during mitosis, and aneuploidy. However, more detailed studies failed to show any link between cohesin mutations and aneuploidy in hematological malignancies (Fisher, 2017; Kon et al., 2013).

Instead, the in-depth sequencing studies revealed five key characteristics of cohesin mutations. (i) the majority (if not all) of cohesin mutations have loss-of-function consequences. And while there are activating mutations in some solid-state cancers (overexpression of RAD21 correlates with poor prognosis in breast and colorectal cancers for instance (Deb et al., 2014; Xu et al., 2011)), in the context of leukemia, cohesin likely has a tumor suppressor function, and a clear correlation between cohesin mutations and prognosis has so far not been shown (Fisher, 2017). (ii) Mutations are generally heterozygous, indicating a haploinsufficient mechanism, although it must be pointed out that STAG2 and SMC1A are located on the X-chromosome, making the mutations functionally homozygous at least in males (and perhaps also in females depending on the level

of chromosome imprinting). (iii) Cohesin mutations show a high variant allele frequency (VAF), indicating that they occur relatively early in leukemogenesis (Jan et al., 2012). (iv) Mutations in cohesin have been linked to CHIP in elderly humans, and because of the relatively high VAF, are likely early contributors to a pre-leukemic state (Genovese et al., 2014; Jaiswal et al., 2014; McKerrell et al., 2015). (v) Lastly, cohesin mutations are mutually exclusive. The mutation status of cohesin genes in AML and MDS have been ascertained for thousands of patients, and of these, only two patient had multiple mutations, where the second one was a missense. Thus, it is unlikely that there is any selective advantage or enhanced leukemogenesis upon additional disruption of the complex. Rather, the opposite seems more plausible; a more severe disruption may inhibit cell function as well as cancer development.

After the discovery of cohesin mutations in leukemia, four papers, including paper III in this thesis, came out within a short time frame, all describing the effects of isolated cohesin deficiency in HSPCs (Mazumdar et al., 2015; Mullenders et al., 2015; Viny et al., 2015). Two of the four studies used murine cells and two used human cells. The strategies to disrupt cohesin were quite different; homozygous/heterozygous knockout, inducible shRNAs *in vivo*, overexpression of leukemia-specific cohesin mutants, and constitutive shRNA expression from lentiviral provirus. Despite this, the results were remarkably similar overall with increased HSC activity, impaired differentiation, and myeloid skewing.

Isolated cohesin deficiency in HSPCs of both species caused increased frequency of MPP and ST-HSCs (LT-HSCs show conflicting results however), and cohesin deficient HSPCs show higher chimerism upon transplantation, harbor enhanced HSC expansion *in vivo*, cause a myeloid skewing *in vitro* and *in vivo*, have an increased serial transplantation capacity as well as increased serial replating potential in CFC assays. Interestingly, while there is a clear preservation of HSPCs upon cohesin deficiency, this does not seem to be the case for more differentiated progenitors; depletion of cohesin in a CMP or GMP for instance has a very mild phenotype, and these cells differentiated quite normally (Mazumdar et al., 2015). Apart from linking cohesin mutations to CHIP, their role in leukemogenesis was functionally proven where haploinsufficiency in Smc3 on a Flt3-ITD background, induced an aggressive AML with decreased latency upon serial transplantation (Mullenders et al., 2015).

Deciphering a clear mechanism of action behind cohesin deficiency has been considerably harder. There are multiple plausibly theories, but it is quite difficult to offer definitive proof, not least due to reasons described in the previous section; the vast heterogeneity of blood cell progenitors. That the mechanism of leukemogenesis is not caused by improper chromosome segregation with subsequent aneuploidy is seemingly clear though.

In mammals, cohesin consists of four core subunits; RAD21, SMC1A, SMC3, and either STAG1 or STAG2 (Peters et al., 2008). These four subunits form a circular structure, capable of encircling chromatin, but they lack direct DNA binding properties, instead relying on various adaptor proteins, CTCF being the most common for DNA localization (Rubio et al., 2008). Apart from the key role of cohesin in mitosis, studies during the last decade have also defined cohesin as a pivotal regulator of the 3D conformation structure of chromatin, and stabilization of chromatin loops, in effect, affecting both replication, transcription, and long-range interactions (Dixon et al., 2012; Ji et al., 2016). With this knowledge, the leading theory has become that cohesin, both independently and together with CTCF, modulates insulation and enhancer interactions within and between preestablished chromosomal architectural compartments and topology associated domains (TADs), thereby influencing gene expression. Additionally, contrary to transcription factors, cohesin remains bound to chromatin during replication (not only at the centromere), suggesting it may play a role in the re-establishment of a certain transcriptional program after mitosis (Yan et al., 2013).

More specifically, in the context of HSPCs, deficiency of cohesin causes global changes in chromosome structure and organization. Overall, the chromatin becomes denser and more compacted, while certain specific loci instead show increased accessibility (Mazumdar et al., 2015; Mullenders et al., 2015; Viny et al., 2015). Mazumdar et al. for instance, showed that overexpression of dominant negative cohesin mutants as well as knockdown of endogenous cohesin caused a relative enrichment in DNA binding motifs for ERG, RUNX1 and GATA2, all known positive regulators of HSC self-renewal. Therefore, a plausible theory is that cohesin deficiency transforms a substantial part of euchromatin to heterochromatin, thus disabling lineage-specific gene activation and blocking differentiation, instead maintaining HSC-specific gene expression programs. Should cohesin be depleted in a more downstream progenitor, where the differentiation program is not only epigenetically primed, but also transcriptionally activated, the effects are negligible (Mazumdar et al., 2015).

Despite the heterogeneity of the mutation landscape of AML and MDS, cohesin has emerged as a major player in both of these diseases where an average of 10-12% of patients harbor mutations in cohesin genes (Fisher, 2017). As a side note, in Down syndrome-acute megakaryoblastic leukemia (DS-AMKL), a subtype of AML, 53% of patients have cohesin mutations (Yoshida et al., 2013). The cause of this overwhelming prevalence is not known though. Clearly, cohesin has become positioned as one of the major gene categories associated with myeloid malignancies.

From a clinical perspective, it is of course very tempting to speculate how cohesin mutations can be exploited for therapeutic benefit, given that a significant number

of patients harbor these mutations. Approaching this problem from the general point of view, with the aim of either restoring function or targeting the defective protein would, in this context, likely prove to be quite difficult. Restoring cohesin function to leukemic cells would require quite advanced gene therapy, and targeting a defective protein is rarely a viable strategy when the disruption is an LOF. Additionally, as cohesin mutations occur early on and leukemia is known to go through several stages of clonal evolution, it is unclear whether established disease is at all dependent on defective cohesin function, bringing even more uncertainty to these two strategies.

An entirely different strategy would be to use the mutual exclusivity of cohesin mutations as a therapeutic advantage, and disrupt cohesin function even further. At first glance, this may seem paradoxical, since defective cohesin correlates with leukemia. However, due to the mutual exclusivity of cohesin mutations, it does seem that mutations in two different core subunits are not tolerated by the leukemic cells. Indeed, a homozygous disruption in just one gene is detrimental to HSPCs as shown by Viny et al., where heterozygous Smc3 deletion causes increased HSPCs activity while homozygous deletion results in aplastic bone marrow, pancytopenia and death (Viny et al., 2015). We have observed a similar dose-dependent effect of RAD21 knockdown in human HSPCs, where partial (40-50%) knockdown induces expansion while near complete knockdown (>90%) results in cell death, further supporting this notion (Galeev, R., and Larsson, J. unpublished data). Targeting the healthy allele of the mutated cohesin gene or one allele of another, healthy cohesin gene, may provide an effective therapy to eradicate leukemic cells.

This concept; synthetic lethality, has already been demonstrated for mutations in splicing genes, commonly mutated in MDS. Like cohesin mutations, they are also heterozygous and mutually exclusive, indicating that only a partial loss of the spliceosomal machinery can be tolerated. Further attenuation of the splicing process in leukemias already harboring mutations in a splicing gene, selectively and strongly perturbed the leukemic cells *in vitro* and *in vivo* (Yoshida et al., 2011).

Should synthetic lethality be pursued in the context of cohesin mutations, great care must be taken not to induce malignancy in healthy cells, since targeting leukemic cells exclusively is quite difficult, not least due to the high morphological similarity, and some level of cohesin disruption would likely be induced in healthy cells as well. Based on sequencing studies, and also our data presented in paper III, STAG1 appears to be the core subunit showing the mildest phenotype, indicating that this gene could be a viable target. Indeed, two recent papers demonstrated synthetic lethality between STAG1 and STAG2, albeit in cancer cell lines (Benedetti et al., 2017; van der Lelij et al., 2017). Nevertheless,

these studies also showed no significant abnormalities upon isolated STAG1 disruption. The key challenge will obviously be developing therapeutically viable and safe strategies of targeting STAG1 in humans.

Nevertheless, the discovery of the role of cohesin in hematopoiesis and leukemia has opened an exciting new avenue for research and development of targeted therapeutics, but also added an additional layer of understanding transcriptional regulation at the epigenetic level in modulation of fundamental hematopoietic cell fate options. In the end, it is a fascinating observation that an evolutionary conserved and general protein complex like cohesin can induce specific gene expression programs in stem cells.

SVENSK SAMMANFATTNING

Bildandet av blodceller, hematopoesen, utgår från ett litet antal av de så kallade hematopoetiska stamceller som sitter i vår benmärg. Dessa celler har förmågan att ge upphov till alla mogna blodcellstyper av de tre hematopoetiska linjerna, samtidigt som de genom celldelning bibehåller sin egna mängd. Det har uppskattats att vi endast har några tusen hematopoetiska stamceller, men trots deras relativt lilla antal, har de den enastående förmågan att generera runt 11 miljoner mogna blodceller varje sekund, hela livet.

Trots att hematopoetiska stamceller sannolikt är de stamceller det forskats mest kring, är vår förståelse kring hur de regleras på molekylär och genetisk fortfarande inkomplett. I syfte att öka vår förståelse om den genetiska regleringen av hematopoetiska stamceller både i normal och malign blodbildning (leukemi), har vi i denna avhandling använt oss av RNA interferens (RNAi) applicerat på blodstamceller från navelsträngsblod i syfte att identifiera gener som styr stamcellernas självförnyelse och celldelning. Genom att använda både ett förvalt och genetiskt komplett RNAi virusbibliotek, har vi funnit att MAPK14, cohesinkomplexet and JARID2 är negativa genetiska regulatorer av blodstamceller. Attenuering av samtliga dessa gener medförde ökad funktionalitet hos stamcellerna.

Vi har också använt datan från vår RNAi screen som ett verktyg att preselektera gener i syfte att även identifiera genetiska regulator i malign hematopoes, särskilt akut myeloisk leukemi och myelodysplastiskt syndrom. Genom att sekvensera de preselekterade generna i över 400 patientprover och därefter funktionellt validera våra upptäckter i både humana och murina celler, har vi identifierat NCAPD2 och SDPR som potentiellt AML- och MDS-associerade gener.

Sammanfattningsvis har vi i denna avhandling använt oss av en global, helgenomtäckande RNAi screen i mänskliga stamceller isolerade från navelsträngsblod. Vi har visat att detta är en användbar metod att hitta nya genetiska stamcellsregulator och i kombination med helgenomssekvensering att även hitta funktionellt relevanta sjukdomsassocierade gener i hematopoetiska maligniteter.

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