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MicroRNA-125a in Murine and Human Hematopoiesis

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MICRORNA-125A IN MURINE AND HUMAN HEMATOPOIESIS

Daniëlle Gaby Luinenburg

The work described in this thesis was conducted at the European Research Institute for the Biology of Ageing (ERIBA) in the laboratory of Ageing Biology & Stem Cells, University of Groningen, Groningen, The Netherlands.

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university of
 groningen

MicroRNA-125a in Murine and Human Hematopoiesis

PhD thesis

to obtain the degree of PhD at the
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on the authority of the
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the decision by the College of Deans.

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Introduction & Outline of the thesis

HEMATOPOIESIS

Blood is a remarkable component of the human body that is both a liquid and a tissue which is essential for the transport of, among other things, nutrients and gasses. Red blood cells transport oxygen and carbon dioxide, white blood cells are part of our immune system and platelets form a safety net to minimize blood loss upon injury. Mature blood cells, like most other mature cell types, have only limited life spans. Red blood cells have a life span of around 120 days, for white blood cells this can range from only a few hours to years.^{1,2} This creates a need for constant replenishment, blood is a highly proliferative tissue. Hematopoietic stem cells (HSCs) fill this need by performing a careful balancing act between self-renewal and differentiation that ensures proper blood function throughout our lives.

Hematopoiesis is the hierarchal process of differentiation of HSCs into all mature blood cell types. (Figure 1) At the top of the hierarchy resides the HSC, which is able to both self-renew and differentiate into multipotent progenitors (MPPs) which in turn can further differentiate into any of the mature hematopoietic cells. Once differentiated it is no longer possible for a hematopoietic cell to move back up the hierarchy and to re-acquire potential that was lost. Differentiation in the hematopoietic system is -to the best of our current knowledge- unidirectional. Therefore, it is essential that the decision process of self-renewal or differentiation of an HSC is tightly regulated.

Figure 1: A simplified representation of differentiation of hematopoietic stem cells (HSCs) into all mature blood cells. MPP: multipotent and pluripotent progenitors; CMP: common myeloid progenitor; CLP: common lymphoid progenitor; GMP: granulocytic and monocytic progenitor; MEP: megakaryocytic and erythroid progenitor.

In a complex interplay, adult hematopoiesis is regulated by both extrinsic and intrinsic factors. The extrinsic stimuli originate from the environment of the HSCs, the bone marrow niche. Supporting cells in the bone marrow secrete cytokines and growth factors that provide signals to the HSCs. A recent study highlights the function of nociceptive nerves, pain receptors, in the bone marrow to regulate hematopoietic stem cell mobilization via the secretion of a peptide that induces granulocyte colony-stimulating factor (G-CSF).³ In addition to these signals, HSCs are regulated by processes that are taking place inside the cell. Such intrinsic factors that regulate the fate of HSCs are controlled at different levels and result in specific gene expression patterns that favor either self-renewal or differentiation. Gene transcription is regulated by epigenetic DNA and histone alterations that determine chromatin accessibility, such that a plethora of transcription factors can initiate expression. After gene expression, post-transcriptional regulatory mechanisms, such as microRNAs, can target mRNAs to alter translation and protein abundance.

Hematopoietic aging

As with any other tissue in the human body the hematopoietic system is affected by the detrimental effects of aging. Aged HSCs are outcompeted by their younger counterparts, their regenerative potential declines, and over time the risk of developing hematological malignancies and immune diseases increases. Although aged HSCs show functional decline, (at least in mice) there is an increase of phenotypically defined HSCs. At the same time the number of actively contributing HSCs to the total blood production decreases,^{4,5} indicating a clear functional decline upon aging. Aged HSCs are still influenced by the bone marrow microenvironment. However, this does not seem to be a main contributor to the aging phenotype. When aged HSCs are transplanted into young recipients, they are still outperformed by their younger counterparts.^{6,7} Cell intrinsic factors are a key element in the functional decline of aged HSCs.

Hematopoietic stem cell expansion

So far, it has been extremely challenging to maintain or efficiently expand HSCs in vitro. Once HSCs are removed from their bone marrow niche, they rapidly lose their self-renewal capacity and start differentiating into different lineages depending, in part, on the cytokines present in the culture media. This differentiation is terminal, it results in cells with a limited lifespan. For stem cell maintenance it is therefore necessary to sustain the self-renewal abilities of these cells. Stimulating self-renewal of HSCs ex vivo will not only ensure maintenance but will also expand the total number of stem cells. This is of significant clinical interest in bone marrow transplantations. For patients in need of an HSC transplant there is limited material available, being able to expand this limited material will increase the chances of a successful transplant. In addition, with the advent of gene therapy approaches to cure hereditary blood cell diseases, methods to maintain stemness during ex vivo perturbations are essential.

MICRORNAS

MicroRNAs (miRs) are small noncoding RNAs of around 22 nucleotides long. The first miR to be identified was lin-4, discovered in 1993 in the nematode *C. Elegans*.⁸ Lin-4 was found to be a key regulator of developmental timing. It took 7 more years to conclude that lin-4 was not alone⁹ and in fact that miRs were also present in other organisms including humans.^{10,11} Shortly after, miRs were also discovered to regulate development of plants in *Arabidopsis*.¹² Since their discovery, the role of mammalian miRs has been shown in the regulation of many processes such as developmental timing, growth control, apoptosis and indeed also in hematopoiesis.^{13,14} Currently, a range of miR databases exist that contain information on evolutionary conservation, expression, experimental and predicted targets of all known miRs.¹⁵⁻¹⁷

MiRs regulate gene expression post-transcriptionally by targeting complementary sequences in the 3'UTR of mRNAs. In plants, this pairing can also occur in 5' UTRs, ORFs and even non-protein-coding transcripts.¹⁸ There are differences between plant and animal miRs in target pairing requirements and biogenesis. For the purpose of this thesis we will focus selectively on animal miRs.¹⁸ In animals, a 6-8 base pair long sequence on the 5' end of the miR - referred to as the seed sequence - is thought to be most important for miR-target interaction. A single miR typically has many different mRNA targets. The miR affects gene expression by interfering with protein translation of the targeted mRNA. MiRs originate from their own genes or from intronic regions of host genes, sometimes called 'mirtrons' (Figure 2).¹⁹ They are further processed by the RNase III type endonucleases Drosha and Dicer. First, the miR precursors are processed in the nucleus by Drosha and later matured in the cytoplasm by Dicer. The mature miRs are then loaded into the RNA induced silencing complex (RISC), which mediates the interaction between miR and their target.^{19,20}

MicroRNA-125

MiRs are indispensable for proper HSC function, as deletion of Dicer causes impaired function of HSCs as a consequence of absence of mature miRs.²¹ In this same study miR-125a was found to control the number of HSPCs by regulating levels of apoptosis.²¹ MiR-125a is part of an evolutionarily conserved cluster consisting of miR-99b, let-7e and miR-125a that is highly expressed in HSPCs. In a genetic screen comparing miR expression in HSCs from two different mouse strains with different stem cell potential, the miR-125a, miR-99b, let-7e cluster was found to be highly expressed in HSC and down-regulated upon differentiation.²² Moreover, the overexpression of both this cluster or miR-125a alone conferred a strong proliferative advantage to HSPCs.²² Further work showed that overexpression of miR-125a confers a strong proliferative advantage to HSCs compared to their wild type counterparts in both murine and human cells.²³ An even more striking effect of miR-125a is its ability to revert hematopoietic progenitor cells back to a more HSC-like state.²⁴ This effect is

Figure 2: An overview of regulation of gene expression by miRNAs. Precursor miRNAs (pri-miR) are transcribed from the genome or spliced from other mRNA transcripts. The pri-miR is processed by proteins Drosha and DGCR8. The resulting pre-miR is exported from the nucleus into the cytoplasm by Exportin5. Maturation of the miR takes place in the cytoplasm where proteins Dicer and TRBP further process the pre-miR into a mature miR. The mature miR is loaded into the RNA-induced silencing complex (RISC) of which the Argonaut proteins 1-4 (AGO1-4) are a main component. The RISC complex mediates the interaction between the miR and its mRNA targets, resulting in repression of translation by stabilization and in some cases degradation of the targeted mRNA.

Image adapted from: Fillipowicz et al. (2008)¹⁷ and Luinburg & de Haan (2020).¹⁸

particularly interesting as it may be used as a tool to restore stem cell potential that has been lost due to premature differentiation. In further studies on the role of miR-125a in HSCs it was also shown that overexpression of miR-125a enhances the clonal lifespan and migration of murine HSPCs.²⁵ These effects of miR-125a on HSC function come with a flipside as persistent overexpression of miR-125a in HSCs has led to myeloproliferative disease.^{22,23}

MiR-125a is part of a family of three members, miR-125a, miR-125b1 and miR-125b2. The three family members are closely related in sequence and all have the same seed sequence in both mouse and human. MiR-125b1 and 125b2 have the same mature sequence but

are encoded by different genomic loci. The three different miR-125 family members have very similar effects on HSCs, when overexpressed they govern a proliferative advantage to HSPCs.²³ Due to these similar effects on HSCs and the identical seed sequence we expect the mechanisms by which these miRs act to be highly similar if not the same. As mentioned before, miRs bind to their mRNA targets by targeting a sequence complementary to the miR's seed sequence. In HSCs, a specific set of miR-125 targets must be responsible for the observed phenotype. In order to further utilize the mechanism by which miR-125 governs its beneficial effect on HSC function it is critical to identify these targeted mRNAs and the proteins they encode for. Only then will we be able to mimic the effects of miR-125 by blocking its targets, enabling the *in vitro* maintenance and expansion of HSCs.

In this thesis we set out to develop methods to reversibly control the expression of miR-125 in HSCs and to identify key targets. Beyond fundamental insight into how miR-125 controls HSC behavior, identification of targets could also allow the development of novel *ex vivo* HSC expansion protocols.

OUTLINE OF THIS THESIS

In **Chapter 2** we review the effect of aging on the function of HSCs and the role that miRs may play in this process. There are several miRs that specifically enhance HSC functions, such as quiescence, self-renewal and differentiation, which have been shown to decline upon aging. By targeting these miRs or miR-targets it might be possible to rejuvenate HSCs.

In **Chapter 3** we describe how we developed and utilize a Cre-LoxP system in order to reverse the overexpression of miR-125a and determine whether its continuous expression is essential for the proliferative phenotype. We show experimental data on the reversion of the overexpression and demonstrate that reversal of the overexpression abolishes the proliferative effects of miR-125a. Finally, we identify novel putative miR-125a targets that affect HSC function.

Chapter 4 focusses on understanding the mechanisms by which miRs bind their targets and which genes might be miR-125a targets in HSCs. First, we focus on the kinetics of miR-target interactions by selectively mutating individual bases, pinpointing which base pairs are essential for functional interaction between miR-125a and their targets. We performed AGO2-RIP-Seq experiments to search for differential binding of mRNA in the RISC complex. Finally, we combine multiple unbiased proteome and transcriptome data sets to search for miR-125a targets in HSCs.

In **Chapter 5** we explore the potential clinical implications of previous research on miR-125a in human cord blood derived HSCs. We further characterize the effects of miR-125a overexpression in a human xenograft model and postulate the potential use of miR-125a in order to expand HSCs for transplantation purposes. In preliminary experiments we also investigate the effects of miR-125a on induced pluripotent stem cell (iPSC) derived hematopoietic cells.

Chapter 6 summarizes our findings, further discusses the implications of this thesis and suggests topics for further research. We discuss various sources of HSCs, targets for the expansion of HSCs and the role that miR-125 can play as a tool to expand HSCs.

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2

MicroRNAs in hematopoietic stem cell aging

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ABSTRACT

The functional decline that is observed in hematopoietic stem cells (HSCs) upon aging is attributed mainly to cell intrinsic factors that regulate quiescence, self-renewal and differentiation. MicroRNAs (miRs) have an indispensable role in the regulation of HSCs and have been shown to also regulate processes related to tissue aging in specific cell types. Here we discuss the role of miRs in the regulation of HSC self-renewal and differentiation throughout life and its implications for future research.

INTRODUCTION

Aging and its detrimental effects on many physiological processes has become a major topic in biomedical research, as life expectancy of the human population is continuously increasing. Important hallmarks of aging encompass a variety of genetic pathways and biochemical processes, including genomic instability, cellular senescence and stem cell exhaustion.¹ The latter is of particular interest in hematology, where the functional decline of aged stem cells manifests in poor tissue function and disease. Hematopoietic stem cells (HSCs) are responsible for the replenishment of all mature blood cell types throughout the entire lifespan of an individual. The maintenance of balance between self-renewal and differentiation of HSCs is tightly regulated but poorly understood. Upon aging, HSCs gradually lose their regenerative potential and show defects when their repopulating potential is tested in transplantation assays. Counterintuitively, whereas the clonal complexity of the hematopoietic system decreases (i.e. blood cell production is derived from fewer and fewer stem cells), at the same time the number of phenotypically defined HSCs increases.²⁻⁴ Thus, although there are more stem cells, their active contribution to blood cell production decreases. Although age-related changes in the bone marrow microenvironment have been described, the origin of this functional decline of HSCs is attributed mainly to cell intrinsic factors that regulate quiescence, self-renewal and differentiation.⁵

MICRORNAS AS REGULATORY COMPONENTS IN AGING HSCS

Intrinsic control of HSC functioning is executed at multiple levels. Firstly, the regulation of gene transcription by transcription factors, guided by epigenetic chromatin and DNA modifications, greatly influences the expression of key stem cell genes. Secondly, after mRNA is transcribed from the genome, multiple factors control the translation of the mRNA into protein. MicroRNAs (miRs) are one of the factors that interfere with gene expression at the mRNA level. As several miRs have been found to play an important role in HSC regulation they are interesting candidates to further study in HSC aging.

MicroRNAs as gene regulatory agents

MiRs are small non-coding RNAs that regulate gene expression mainly by targeting the 3' untranslated region (UTR) of mRNAs present in the cytosol. The primary transcript of a miR can originate from an isolated miR gene or from the intronic region of another (host) gene's transcript. After processing by Dicer and Drosha a mature miR remains, which is loaded into the RNA-induced silencing complex (RISC). Mature miRs are typically ~22 base pairs long and one miR often has many different downstream mRNA targets. Physical interaction between a miR and its mRNA targets induces cleavage of the mRNA or blocks the translation of the mRNA into protein.^{6,7} In this way, miRs are able to fine-tune the abundance of different cellular proteins. (Figure 1)

Figure 1: Intrinsic regulation of HSC gene expression by miRs.

Intrinsic regulation of HSCs takes place at different levels. The regulation of gene transcription by epigenetic modifications and transcription factors controls the expression of different genes. After mRNA is transcribed, miRs can alter protein abundance by inhibiting the translation of their target mRNA into protein by translational repression or endonucleolytic cleavage which results in degradation. Precursor miRs (pre-miR) are transcribed from their own gene or spliced from an intronic region of another gene. After maturation, the miR is loaded into the RNA-induced silencing complex (RISC) which mediates the interaction between miR and target mRNA.

MicroRNAs as hematopoietic regulators

Soon after their discovery in *C. elegans* it became clear that miRs have an indispensable role in regulation of development and stem cells.⁸ Where some miRs are general regulators of development, like let-7 in *C. elegans*, other miRs have shown to have highly tissue-specific effects. In the mammalian hematopoietic system, multiple distinct miRs have been found to have specific effects on the behavior of stem cells upon perturbation of their expression. While some miRs, such as miR-181, miR-223 and miR-142s induce differentiation of HSCs⁹, the opposite is true for miR-23a, as deletion of this gene results in increased differentiation, suggesting that this miR blocks differentiation. However, when deletion of miR-23a

is combined with deletion of the closely related miR-23b, this leads to an overall reduction in hematopoietic stem and progenitor cells (HSPCs).¹⁰ These examples, combined with the fact that miRs have many different mRNA targets, show the intricacy of miR networks that regulate HSCs.

MicroRNAs that regulate quiescence and self-renewal of HSCs

MiRs form intricate networks that regulate many processes in diverse tissues. In HSCs, a limited number of miRs has been found to be specifically associated with maintenance of their quiescence or expansion by targeting various different mRNAs and pathways. Several key examples are listed in table 1. These miRs are of main interest because they actively stimulate the expansion of HSCs whilst at the same time maintaining their unique stem cell properties. These miRs may therefore be candidates to further investigate their potential use as an HSC expansion- or function-restoring agent. A particularly interesting example is miR-132, which directly targets FOXO3, buffering the increasing expression of this aging related transcription factor and keeping HSCs in a more quiescent state.

MicroRNAs in aging and age-related hematopoietic dysfunction

Advanced age is associated with a progressive functional decline in many, if not all, tissues. The transcriptome of cells in an aged tissue changes when compared to their younger counterparts, suggesting a role for miRs. Indeed, many reviews highlight the role of miRs in diverse age-associated processes and diseases, such as senescence, Alzheimer's disease, metabolic changes and the regulation of epigenetic changes.^{24–27} For example, in Alzheimer's disease, it has recently been shown that miR-298 and miR-455-3p protect against abnormal amyloid precursor protein processing by targeting the 3'UTR of the mRNA of this protein.^{28,29}

There is a plethora of miRs that are associated with many different cancer types through various pathways. These miRs are also referred to as oncomiRs. In the aging hematopoietic system, the risk of leukemia increases with age. In these age-related leukemias many studies have identified miRs that are preferentially expressed in leukemic (stem) cells, suggesting that they contribute to disease. A particularly interesting example is miR-146b, which targets the NF- κ B pathway. Deletion of this miR leads to the sustained activation of this pathway and results in the development of hematopoietic malignancy upon aging in mice.³⁰

MiR-29 and miR-101 can regulate epigenetic changes by targeting proteins that are part of the epigenetic machinery, such as DNA methyltransferases 3A and B or the Polycomb Repressive Complex 2 protein EZH2.^{31,32} MiR-29a targeting of DNMT3A induces the maintenance of HSC self-renewal.¹³ Interestingly, mutations in DNMT3A are strongly associated with age-related clonal hematopoiesis, and are considered to lead to the emergence of a

preleukemic stem cell clone.³³ Clonal hematopoiesis arises from the preferred outgrowth of an HSC which has acquired a somatic mutation in one of a very restricted number of genes that often encode for proteins involved in epigenetic regulation. A single mutant HSC can give rise to a disproportional amount of progeny, and this phenomenon is strongly age-dependent and occurs in a significant fraction of healthy elderly individuals. Interestingly, some of the targets listed in table 1, DNMT3A and TET2, are also associated with clonal hematopoiesis.³⁴ The epigenetic regulators DNMT3A and TET2 are not only highly prevalent mutations in clonal hematopoiesis, but also influence inflammatory responses of mature cells derived from the mutated HSCs. This indicates that miRs also may have the potential to influence a specific aging phenotype such as clonal hematopoiesis.

Table 1: Examples of miRs that are specifically associated with maintenance of quiescence or expansion through various different targets and pathways.

microRNA	Effect on HSCs	Target	Pathway	Species*
21 ₁₁	Maintains HSC homeostasis	PDCD4	Inflammation	M
22 ₁₂	Regulates HSC maintenance and self-renewal	TET2	Epigenetic	M
29a ₁₃	Maintains self-renewal	DNMT3A	Epigenetic	M
99 ₁₄	Inhibits differentiation and cell cycle entry	HOXA1	Development	H
125a/b ₁₅	Induces long-term repopulating abilities in progenitors	MAPK14 (P38)	Inflammation	M/H
126 ₁₆	Drives quiescence and self-renewal in leukemic stem cells	CDK3	Cell cycle	H
127-3p ₁₇	Limits differentiation	NA		M
132/212 ₁₈	Regulates HSC maintenance and survival with age	FOXO3	Transcription factor	M
139-5p ₁₉	Regulates proliferation in early hematopoiesis	BRG1	Epigenetic	M/H
143/145 ₂₀	Depletion of HSCs, activation of progenitors	TGFβ	Growth factor	M
155 ₂₁	Promotes G-CSF induced mobilization	CXCL12	Growth factor	M
193b ₂₂	Controls HSPC expansion	c-KIT	Receptor	M
382-5p ₂₃	Supports expansion of granulocyte lineage	MXD1	Transcription factor	H

*Species: M=*Mus musculus*; H=*Homo sapiens*

MICRORNA-BASED INTERVENTIONS IN AGING HSCS

Old HSCs display aberrant quiescence, self-renewal and differentiation. MiRs that are able to regulate these processes therefore might be able to restore the homeostasis to the ‘young’ condition. In this way, miR interventions could potentially be used as a tool to rejuvenate HSCs. Examples of anti-aging interventions that come to mind include the mTOR inhibitor rapamycin and caloric restriction. For the latter there is no evidence that dietary interventions prevent HSCs aging.³⁵ Rapamycin on the other hand might have beneficial effects on aging HSCs.³⁶ Rapamycin inhibits the serine/threonine protein kinase mTOR, a regulator of cell growth, metabolism and autophagy. From the miRs highlighted in table 1, miR-21, miR-22, miR-99, miR-125a/b and miR-155 are all found to target genes in the mTOR pathway.³⁷

MicroRNAs as rejuvenation tools

In cardiomyocytes it has been shown that the intra-cardiac injection of miR-19a/19b mimics enhances cardiomyocyte proliferation and stimulates cardiac regeneration in response to myocardial infarction injury.³⁸ In HSCs, miR-125a may qualify as an HSC rejuvenating factor. Ectopic expression of miR-125a in HSCs results in very potent stem cells with an extended lifespan of individual clones. Moreover, expression of miR-125a in progenitors reverts these cells to a stem cell like phenotype and enables these progenitors to self-renew (a characteristic that they normally lack) and to constitute long-term engraftment. This phenotype was first discovered in murine cells, but is highly evolutionary conserved and also confirmed in human cord blood derived HSCs.³⁹ Potentially, the addition of miR-125a mimics to an HSC in vitro culture or even local administration in vivo, could enhance HSC function and functionally rejuvenate old cells by repressing key target genes.

Target identification and manipulation

Another approach to improve HSCs function may be the direct inhibition of essential miR targets. A single miR has many different mRNA targets, which are typically part of inter-linked pathways of proteins or other non-coding RNAs. It is therefore possible to directly inhibit the protein downstream of the miR, as opposed to mimicking the miR itself. This may circumvent some of the issues that come with miR mimics as discussed below. Central to this approach is the notion that targets of candidate miRs are identified, which has turned out to be difficult. Different databases exist that use specific algorithms to predict miR targets. An example is MirTarget, which predicts targets based on the analysis of existing data of miR-target interactions from sequencing experiments.^{40,41} These tools are very useful; however, they often do not provide experimentally validated or tissue specific targets. Different experimental techniques are established for the discovery of indirect and direct targets of miRs, each with their own advantages and caveats.⁴² Once the regulatory network linked to a miR of interest is pinpointed, this will open up possibilities for direct inhibition of miR targets and mimicking the positive effects that miRs can have on aging HSCs.

FUTURE PERSPECTIVES

Just as a single aging gene does not exist, there is also not a single microRNA that is solely responsible for the changes observed upon aging. Rather, it may be informative to search for an overall tissue-specific change in miR-profiles associated with aging. Some of these miRs will play a role in more general processes that are shared between different tissues, and some miRs will be linked to specific tissue properties. In the case of HSC aging, differentially expressed miRs will most likely be miRs that are directly linked to stem cell functioning such as self-renewal and differentiation.

Multiple mechanisms are responsible for the functional changes that are observed in the hematopoietic system upon aging. As miRs have been shown to play important roles in HSC self-renewal, it seems likely that miRs are responsible for at least part of the aging phenotype in HSCs. At present, however, our understanding of miR-regulated HSC aging remains poor. MiRs that specifically regulate HSC quiescence and self-renewal would be interesting candidates to further explore with regards to their potential as a rejuvenation tool. In order to identify potential deregulated single miRs or miR networks an effort should be made to systematically screen for these changes in young and old hematopoietic stem cells. MiR-knock-out models that allow for conditional knock-out of miRs can be a valuable resource.⁴³

Interference in miR activity could be achieved by mimicking the effects of a miR using RNA interference (RNAi) based techniques, however these still come with technical limitations that need to be resolved in the future. In vivo use of miR mimics is hampered by the stability and delivery of these therapeutics.⁴⁴ Another issue that will need to be resolved is the activation of the immune system that is inherently linked to the utilization of the RNA interference (RNAi) mechanism, which is indispensable for the cell's defense against invading double stranded RNA (dsRNA) and abnormal DNA. Effectively identifying miR regulatory networks in aging cells and tissues, by characterizing and validating mRNA targets, may offer opportunities to manipulate cells, both in vitro and in vivo.

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CONFLICT OF INTEREST DISCLOSURE

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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3

Persistent expression of microRNA-125a is required to induce murine hematopoietic stem cell repopulating activity

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ABSTRACT

MicroRNAs (miRs) are small non-coding RNAs that regulate gene expression post-transcriptionally by binding to the 3'UTR of their target mRNAs. The evolutionarily conserved microRNA-125a (miR-125a) is highly expressed in both murine and human hematopoietic stem cells (HSCs) and previous studies have shown that miR-125 strongly enhances self-renewal of HSCs and progenitors. In this study we explored whether temporary overexpression of miR-125a would be sufficient to permanently increase HSC self-renewal, or rather whether persistent overexpression of miR-125a is required. We used three complementary in vivo approaches to reversibly enforce expression of miR-125a in murine HSCs. Additionally, we interrogated the underlying molecular mechanisms responsible for the functional changes that occur in HSCs upon overexpression of miR-125a. Our data show that continuous expression of miR-125a is required to enhance HSC activity. Our molecular analysis confirms changes in pathways that explain the characteristics of miR-125a overexpressing HSCs. Moreover, it provides several novel putative miR-125a targets, but also highlights the complex molecular changes that collectively lead to enhanced HSC function.

Highlights:

- Continuous expression of miR-125a is required to induce self-renewal of HSCs
- Cre-LoxP excision is a reliable tool for reversal of microRNA expression in HSCs
- Novel miR-125a associated genes that affect HSC function

INTRODUCTION

Hematopoietic stem cells (HSCs) are responsible for the lifelong reconstitution of all mature blood cells. As differentiation of these cells is non-reversible and terminal, the balance between self-renewal and differentiation of HSCs is tightly regulated to ensure proper tissue function. Proper HSC function is regulated both extrinsically by environmental factors that are secreted by the supporting cells in the bone marrow known as the stem cell niche, and by stem cell intrinsic programs. One of these cell-intrinsic regulatory components that control HSC function are microRNAs (miRs).^{1,2} These ~22 nucleotide long non coding RNAs are integrated into RNA-induced silencing complexes (RISC) that mediate the interaction between miRs and their target mRNAs. After this interaction the translation of mRNA into protein cannot be completed.

MicroRNA-125a is highly expressed in HSCs and its expression declines upon differentiation into more mature cell types further down the hematopoietic lineage.³ MiR-125a is part of an evolutionarily conserved cluster, which also encodes miR-99b and let-7e. The miR-125 family consists of three paralogs: 125a, 125b1 and 125b2 and has an important function in the regulation of the output and persistence of HSCs^{1,4}. Mir-125b1 and mir-125b2 do not differ in sequence but are encoded by two separate loci. All three members of the miR-125 family have similar effects on HSCs when overexpressed.⁵ Due to the high evolutionary conservation, all mature miR-125 family members are present in organisms ranging from nematodes to mammals⁶, and have the same seed sequence in both mouse and human. We will refer to miR-125 to include all three or any of those family isoforms.

Collectively, studies from multiple labs have convincingly demonstrated the unique ability of miR-125 to regulate HSCs.^{1,3-5,7,8} Upon ectopic overexpression of miR-125a, the self-renewal capacity of HSCs is increased in both murine and human cells.⁸ Remarkably, miR-125a is also able to confer long-term repopulating ability upon committed progenitors which they normally lack.⁷ Using cellular barcoding we have recently shown that miR-125a is able to extend the longevity of HSC clones.⁸

Whereas it has become evident that miR-125 affects the activity of targets that need to be repressed to ensure HSCs self-renewal and engraftment, it has remained unclear whether the continuous activity of miR-125 is required, or rather whether temporary overexpression of miR-125 in HSCs is sufficient to induce their long-term self-renewal. In this study we therefore used a reversible expression system (in three different setups) to assess to what extent temporary activation of miR-125a is sufficient to increase the potential of HSCs. We show that reversal of miR-125a overexpression reverts HSCs back to their normal state. Thus, continuous expression of miR-125a is needed to induce self-renewing HSC activity. In addition to functional assays, we have performed transcriptome analysis of HSPCs in

which miR-125a is overexpressed. We identify affected molecular pathways that explain the phenotype that is induced upon miR-125a overexpression and identify novel HSC-specific miR-125a associated gene expression changes.

METHODS

Mice - Female C57BL/6.SJL (B6.SJL, CD45.1) donor mice were bred at the Central Animal Facility of the University of Groningen. Female C57BL/6 (B6, CD45.2) recipient mice were purchased from Envigo (Horst, the Netherlands) and housed under clean conventional conditions. All animal experiments were approved by the Groningen University Animal Care and Use Committee.

Retroviral transduction - Cells were pre-stimulated for 24 hours in StemSpan (Stemcell Technologies, Vancouver, Canada) supplemented with 10% FCS, 1% Penicillin-Streptomycin and cytokine mix: SCF (Stem Cell Factor, 300ng/mL), Flt3 ligand (1ng/mL) and IL11 (20ng/mL). Viral supernatant was produced by transfecting the Platinum-Eco cell line with Fugene HD Transfection Reagent (Promega, Wisconsin, US) according to manufacturer's protocol and harvesting fresh virus 48 hours after transfection. Viral particles were bound to Retronectin (Clontech, Kyoto, Japan) coated plates according to the manufacturer's protocol. After pre-stimulation, HSCs were harvested, added to the virus-coated plates and spun down at room temperature at 400 g for 45 minutes to promote binding of the cells to the virus coated plate.

Primary transplants - Donor cells were harvested from C57BL6/SJL (CD45.1) mice and sorted by FACS for the LSK/SLAM (lineage-Sca1⁺cKit⁺CD48⁻CD150⁺) phenotype. Recipient mice (B6, CD45.2) were conditioned by a lethal dose (9 Gy) of total body irradiation. Transduced cells were transplanted at different dosages in the presence of 2 million supporting cells from a cKit deficient (W41) donor by retro-orbital injection. Ciprofloxacin was added to the drinking water of recipient mice for 10 days, starting 1 day before irradiation. Donor engraftment was tracked by performing retro-orbital peripheral blood sampling every 4 weeks. Erythrocytes were lysed with ammonium chloride-based lysis buffer and remaining white blood cells were stained for CD45.1/2 and markers Mac-1/Gr-1, B220 and CD3. Both stem cell and peripheral blood staining protocols are described in supplementary table S3 and S4.

Serial transplants - Donor cells were harvested from primary/secondary recipients from the miR-125a overexpression and control groups. Total bone marrow was stained for the LSK/SLAM (lineage-Sca1⁺cKit⁺CD48⁻CD150⁺) markers for analysis, however sorting was only performed based on the lineage and Sca1 markers (Figure S1). We have previously described how cKit is a target of miR-125a, resulting in unreliable expression of this stem cell marker in miR-125a overexpressing cells.⁸ Sorted lineage-Sca1⁺GFP⁺ were transduced with the

self-inactivating Cre plasmid⁹ according to the described transduction protocol, and either transplanted directly into secondary recipients or subjected to in vitro expansion. Separation of GFP⁺ and mCherry⁺ stem cells took place by sorting bone marrow from secondary recipients or in vitro expansion (Figure S2). GFP⁺ and mCherry⁺ cells originating from the same bone marrow sample were transplanted in equal dose to ensure comparability.

Cre-loxP system - The MXW-pPGK- IRES-eGFP retroviral vector used for overexpression of miR-125a was constructed as previously described.^{3,5} The first LoxP sequence was inserted directly after the pPGK promotor region by using the XhoI and EcoRI restriction sites. (Oligo sequence: (+) TCGAG ataactcgtatagcatacattatagaagttatGTATGG, (-) AATTCATACata-acttcgtataatgtatgctatacgaagttatC). The second LoxP sequence was inserted behind IRES-eGFP using the SalI and NotI restriction sites. (Oligo sequences: (+) GGCCGCataactcgtatagcatacattatagaagttatAGATAG, (-) TCGACTATCT ataactcgtataatgtatgctatacgaagttatGC) Finally, the mCherry sequence was amplified from a donor vector (pLM-CMV-R-Cre) with primers that included a STOP codon insertion, and inserted behind the second LoxP site. (Forward: TAAGCAGTCGACatggtgagcaagggcgagga, Reverse: tgcttaGTCGACTTA ctgtacagctcgtc-catgccg) This resulted in a plasmid that contains the miR-125a genomic sequence linked to IRES-eGFP flanked by two LoxP sites (floxed) followed by mCherry (Figure 1A). The LoxP sites allow for Cre-recombinase mediated excision and thus shut down of miR-125a overexpression, while at the same time switching the fluorescent tag from GFP to mCherry. Cre-recombinase expression is established by transducing the target cells with a self-inactivating Cre expression plasmid⁹ according to the transduction protocol described above.

In vitro expansion of HSCs - Culture conditions that were described in Wilkinson, A. C. et al.¹⁰ were adapted to suit the needs of this experiment. Culture medium was Ham's F12 (Gibco, Invitrogen, Waltham, Massachusetts, US) supplemented with: 1% Penicillin/Streptomycin/Glutamine (P/S/G), 10mM HEPES, 1mg/ml Polyvinyl alcohol (PVA) and Insulin-Transferrin-Selenium-Ethanolamine (ITSX), plus cytokines: 10ng/ml stem cell factor (SCF) and 100ng/ml thrombopoietin (TPO). Cells were kept in Retronectin coated 6-well plates as described for transduction, cells were seeded at $0.5 - 1 \times 10^6$ cells/well and daily 90% medium changes were performed to eliminate cell debris, metabolites and inflammatory cytokines and ensure constant culture conditions.

Cobblestone assays - The cobblestone-area forming cell (CAFC) assay was performed as previously described.¹¹⁻¹³ In short: hematopoietic cells are seeded onto a feeder layer (FBMD-1) in 96 well plates in a limiting dilution fashion. Wells are scored positive or negative for formation of cobblestone areas and 50% of media is refreshed weekly. Early emerging colonies (day 7-14) are indicative of progenitor activity, late emerging colonies (day >35) are indicative of activity of more quiescent stem cells. The Extreme Limiting Dilution

Analysis (ELDA) tool from the Walter & Eliza Hall Institute of Medical Research¹⁴ was used to estimate the stem cell frequencies and test differences between the experimental groups.

RNA sequencing - Freshly isolated BM lineage⁻Sca1⁺GFP⁺ cells were sorted as shown in Figure S2. 2000 cells were directly sorted into PCR tubes containing 6 μ L NEBNext Cell Lysis Buffer supplemented with RNase inhibitor buffer, immediately frozen in dry ice and stored at -80 awaiting library prep. Library prep was performed according to NEBNext single cell/low input RNA library prep kit for Illumina (New England Biolabs, Ipswich Massachusetts, US) according to manufacturer's protocol. Three control and three OE samples were pooled in equimolar fashion and sequenced on Illumina NextSeq500 platform. Demultiplexed sequencing reads were subjected to quality control and mapped to the murine genome (version M21) with STAR aligner optimized for paired end reads of 50 bp. Differential expression analysis was performed using edgeR, in brief: low read counts were removed, normalization method TMM was applied. One sample of each experimental group was removed. Further, glmQLFit/glmLRT model was used to fit the data. Differentially expressed genes were selected with topTags function, using Benjamini-Hochberg adjustment and the significance threshold at $p < 0.05$. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus¹⁵ and are accessible through GEO Series accession number GSE158126 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE158126>).

RESULTS

We evaluated the effects of reversal of miR-125a overexpression on the functioning of HSCs using three different *in vivo* approaches. Firstly, we performed competitive transplantation studies in which miR-125a overexpressing cells were transplanted alongside HSCs in which miR-125a overexpression was reversed (Figure 1). In a second set of experiments, we performed non-competitive transplantations where miR-125a reversed cells were transplanted into recipients without the competitive pressure of cells that still overexpressed miR-125a (Figure 2). Finally, we expanded HSCs in which miR-125a was overexpressed and then deleted *in vitro*, and compared their engraftment activity non-competitively (Figure 3).

Using Cre-LoxP recombination to reverse miR-125a overexpression in hematopoietic cells

In order to investigate whether the potent effect of miR-125a on HSCs requires its constitutive expression, we utilized the Cre-LoxP system to generate a reversible overexpression system that allows to track cells in which miR-125a expression is induced and simultaneously labels cells in which miR-125a expression has been reversed. To this end we generated a retroviral vector, based on the vector previously used for miR-125a overexpression⁵, and added LoxP palindromic sites and a second fluorescent marker (Figure 1A). This allows

for Cre-recombinase mediated reversal of miR-expression accompanied by switching of a stably integrated fluorescent marker (Figure 1B). Upon miR-125a overexpression cells are marked by GFP (green fluorescent protein), at any desired time these cells can be reverted back to endogenous expression levels of miR-125a which then simultaneously marks these reverted cells with mCherry (red fluorescent protein). The reversal of miR-125a expression back to endogenous levels is apparent 48 hours after Cre-recombinase activation and is accompanied by expression of the red fluorescent protein mCherry (Figure 1C). FACS analysis indicates that the switch of fluorescent labels from GFP to mCherry requires 5 days to be fully completed (Figure 1D).

Figure 1: Set-up and kinetics of Cre-LoxP reversible miR expression system. (A) Schematic representation of the retroviral plasmid, both for 125a OE and the empty vector control. (B) MiR-125a expression levels upon overexpression and reversal in 32D cell line. (C) Overview of reversal of overexpression by Cre recombinase resulting in a switch of fluorescent proteins. (D) Kinetics of reversal, change in expression shown as fraction of expression on day 0. Fluorescence measured by flow cytometry, miR-125a levels measured by RT-qPCR.

Continuous overexpression of miR-125a is required to evoke the proliferative advantage of HSCs

Using this reversible expression system, competitive serial transplants were performed in order to establish whether continuous expression of miR-125a is required to increase HSC potency (Figure 2A). Here we use the term competition to indicate the interplay between the two subpopulations in the donor pool; the miR-125a overexpressing GFP⁺ cells and the reversed mCherry⁺ cells. The transplantation protocol included a lethal dose of irradiation and the use of W41 supporting cells which provide short-term radioprotection but no long-term engraftment. First, donor cells were harvested from B6.SJL (CD45.1) mice and miR-125a overexpression was induced by viral transduction of the reversible expression vector. HSCs were then transplanted into lethally irradiated B6 (CD45.2) recipients and allowed

to engraft for 14 weeks. After 14 weeks the bone marrow was harvested and, as expected, a strong engraftment of the miR-125a overexpressing cells was observed (Figure 2B). Reversal of miR-125a expression was subsequently induced in vitro using viral transduction of a self-inactivating Cre recombinase expression vector, and cells were immediately transplanted competitively into secondary recipients. The contribution of GFP⁺ and mCherry⁺ cells to the peripheral blood was monitored at various time intervals (Figure 2C). In such a competitive setup, reversed cells were outcompeted by miR-125a overexpressing cells in 2 weeks.

Figure 2: Continuous overexpression of miR-125a is necessary for the proliferative advantage phenotype. (A) Experimental set up. (B) Contribution of GFP⁺ cells to the total white blood cell (WBC) population in bone marrow (BM) and spleen of mice transplanted with cells transduced with an empty vector (EV) or with miR-125a overexpression, $n = 3$ mice/group. (C) Contribution over time of CD45.1⁺GFP⁺ and CD45.1⁺mCherry⁺ cells to the peripheral blood of secondary recipients.

MiR-125a reversion does not negatively affect HSC function

In the previous experiment, reversed and non-reversed cells were in competition with each other. It is clear that GFP⁺ cells that still overexpress miR-125a, outcompeted the reversed mCherry⁺ cells. From that experiment it was not clear whether mCherry⁺ cells were exhausted or whether these cells still possessed HSC potential but were outcompeted by the highly competent HSCs that were still overexpressing miR-125a. Thus, in an alternative approach, HSCs in which miR-125a overexpression was reverted were transplanted non-

competitively. This approach allowed to assess their function without the potent competition of miR-125a overexpressing cells (Figure 3A). Similar to the experiment described above and outlined in Figure 2A, cells were harvested from CD45.1⁺ donor mice, transduced with the reversible expression vector and transplanted into lethally irradiated CD45.2⁺ primary recipients. Contribution of GFP⁺ miR-125a overexpressing cells (Figure 3B), total white blood cell counts (Figure 3C) and donor chimerism (Figure 3D) was measured in peripheral blood. After 14 weeks, bone marrow of the primary recipient was harvested and reversal of miR-125a OE was induced by Cre-recombinase expression. Unfractionated cells were then immediately transplanted into a secondary recipient, in order to ensure proper maturation of the fluorescent proteins. Bone marrow of the secondary recipients was harvested after 2 weeks and GFP⁺ and mCherry⁺ were separated by FACS and non-competitively transplanted in limiting dilution into tertiary recipients. In the tertiary recipients, the contribution to the peripheral blood was measured by total donor engraftment (Figure 3E and F), and stem cell frequencies were estimated based on transplantation dose and engraftment. Stem cell frequencies in both miR-125a overexpressing and reversed cells were ~10 fold higher compared to the empty vector control samples, and there was no significant difference between the miR-125a overexpressing and reversed groups ($p=0.056$) (Figure 3G). Progeny of miR-125a overexpressing HSCs showed myeloid skewing, as reported previously^{5,7}, which is also visible in the peripheral blood of tertiary recipients at week 10 (Figure 3H) and week 18 post-transplant (Figure 3I). Interestingly, this skewing of lineage distribution was persistent in the reverted mCherry⁺ cells in the miR-125a group. These experiments revealed that after reversal of miR-125a overexpression HSCs have a similar stem cell frequency and are still able to engraft and contribute to the peripheral blood.

Different experimental setups yield similar results, persistent expression of miR-125a is required for enhanced HSC function

Serial transplants induce significant proliferative stress on the transplanted HSCs. As an alternative approach to assess the effects of miR-125a reversibility we eliminated secondary transplantations and instead used a recently described 6 days in vitro expansion protocol.¹⁰ After in vitro culture, we again separated the GFP⁺ cells from the mCherry⁺ cells by FACS and assessed HSC function by cobblestone area forming cells assays (CAFC) and by transplantation (Figure 4A). After 6 days of in vitro expansion, cells were analyzed for expression of lineage and stem cell markers (Figure 4B-C). The cultured cells remained lineage negative and showed a similar LSK and LSK/SLAM phenotype in the different experimental conditions. CAFC assays revealed that the miR-125a overexpressing GFP⁺ cells maintained the highest frequency of late appearing CAFCs. Within the EV control groups, no difference was observed between the GFP⁺ and reversed mCherry⁺ cells, as expected. At week 2 the average number of CAFC/10³ cells for EV-GFP, EV-mCherry, 125a-GFP and 125a-mCherry are 3.40, 1.46, 8.23 and 0.99, respectively. At week 5 the average number of CAFC/10³ cells

Figure 3: Reversed cells are still functional in non-competitive transplant. (A) Experimental set up. (B) CD45.1⁺GFP⁺ cells in peripheral blood of primary recipients, $n = 6-8$ mice/group. (C-D) WBC counts and donor contribution (CD45.1⁺) in peripheral blood of primary recipients, $n = 6-8$ mice/group. (E-F) Donor contribution of reversed cells to the peripheral blood in mice transplanted with EV control cells and miR-125a OE cells in tertiary transplants, $n = 4-6$ mice/group. (G) Estimated stem cell frequencies based on transplantation dose and engraftment of tertiary recipients, calculated with ELDA tool.¹⁴ Recipients were marked as positive if $> 1\%$ donor contribution was present in total living cells of bone marrow. Data are shown as 95% confidence interval of $1/(\text{stem cell frequency})$. Pairwise differences tested by Chi-squared test, statistically significant difference between 125a-mCherry and EV-GFP/EV-mCherry, p values respectively: 0.0009 and 0.0013. (H, I) Lineage distribution shown as % of CD45.1⁺ donor cells in peripheral blood of secondary recipients at week 10 (H) and week 18 (I) post-transplant. GM = Granulocyte/macrophage (Gr1/Mac1), T = T-cells (CD3), B = B-cells (B220). Normalized to sum of lineage⁺ cells (Gr1/Mac1/CD3/B220 positive), $n = 2-3$ mice/group.

Figure 4: Reversed cells behave like normal stem cells in vitro. (A) Experimental set up. (B) Cell surface phenotype of lineage⁺Sca1⁺ cells exposed to 6 days in vitro culture. Shown are the LSK and LSK/SLAM compartments. (C-D) Cobblestone area forming cell frequency per 1000 seeded cells shown per week after initiation for respectively EV and miR-125a OE cells.

for EV-GFP, EV-mCherry, 125a-GFP and 125a-mCherry were 42.4, 27.7, 130.6 and 21.3, respectively. Between the two replicates of the miR-125a groups, an average decrease in early appearing progenitor colonies at week 2 (day 14) of ~8-fold and a ~6-fold decrease of late appearing stem cell colonies at week 5 (day 35) was observed upon reversal of miR-125a overexpression.

The various cell populations (HSCs that still overexpress miR-125a, HSCs that overexpressed miR-125a in primary recipients, and the empty vector control cells) were all transplanted non-competitively into secondary recipients at different doses (Figure 5B). In these experiments miR-125a-GFP⁺ cells outperformed control cells, as well as 125a-mCherry cells that no longer overexpress miR-125a, as shown by a higher contribution of these cells to the peripheral blood of recipients over time (Figure 5A). At week 12 there is a clear difference in donor contribution to the peripheral blood (Figure 5C). Upon sacrifice at week 22 post-

transplant, we measured engraftment in the bone marrow and found high chimerism levels in mice transplanted with miR-125a overexpressing cells. This indicates that HSCs that still overexpress miR-125a will outperform reversed and empty vector control cells (Figure 5D). In the bone marrow, the same lineage skewing was visible as seen in the peripheral blood in the previous experiment (Figure 3 H-I). We observed skewing towards the myeloid lineage in the progeny of both miR-125a overexpressing and reversed HSCs (Figure 5E).

Figure 5: Donor contribution of in vitro expanded cells measured in the peripheral blood of secondary recipients. (A) Donor contribution (%CD45.1) of total white blood cells (WBC), $n = 4-5$ mice/group. (B) Number of transplanted lin⁺Sca1⁺ cells. (C) Donor contribution (%CD45.1) in peripheral blood of individual recipients ($n=5$) at week 12 post-transplant. Difference between 125a-GFP and 125a-mCherry tested by paired t-test ($p = 0.0022$) (D) Donor contribution (%CD45.1) in bone marrow of secondary recipients at week 22 post-transplant. (E) Lineage distribution shown as % of CD45.1⁺ donor cells in bone marrow of secondary recipients at week 22 post-transplant. GM = Granulocyte/macrophage (Gr1/Mac1), T = T-cells (CD3), B = B-cells (B220). Normalized to sum of lineage⁺ cells (Gr1/Mac1/CD3/B220 positive), $n = 2-4$ mice/group.

Identification of microRNA-125a associated transcripts by RNA sequencing

In order to provide insight into the molecular mechanisms by which miR-125a exerts its function we established the transcriptome changes upon expression of miR-125a using RNA-sequencing. For this purpose, 2000 lineage⁺Sca1⁺GFP⁺ miR-125a overexpressing or EV control cells were isolated from primary recipients 12 weeks after transplantation. At

this time HSCs have had time to recover from the transplantation procedure and return to a steady state. Direct effects of miRs on their target mRNA could result both in a decrease or an increase of mRNA abundance. Potentially, a miR could also stabilize mRNA abundance in which case we would not be able to identify these transcripts with this approach. Differential expression analysis revealed 156 downregulated and 237 upregulated genes. Comparison of differentially expressed genes with predicted miR-125a targets (compiled from four commonly used sources: PicTar, TargetScan, miRanda and miRDB), allowed us to identify 12 predicted targets that were downregulated upon miR125a overexpression, but we also identified 19 predicted miR-125a targets that were upregulated (Figure 6A-B).

Figure 6: Identification of predicted targets of miR-125a by RNA-sequencing. (A) Scatterplot of gene expression, showing log fold change (logFC), $-\log_{10}$ false discovery rate ($-\log_{10}$ FDR) and expression levels expressed as the log count per million (logCPM). A predicted miR-125a target list was compiled from four commonly used databases: PicTar, TargetScan, miRanda and miRDB. Genes occurring in both the compiled predicted miR-125a target list and significantly differentially expressed are annotated with their gene name, downregulated genes in green and upregulated genes in orange. (B) Overlap between the number of downregulated genes (Down), upregulated genes (Up) and the compiled predicted miR-125a Target list. Hypergeometric probability of overlap Down+Target $p=0.10$, Up+Target $p=0.067$, assuming a total population of 17039, representing all annotated genes in DE table generated from RNA-seq data.

Table 1 provides an overview of the 12 predicted miR-125a target genes that were significantly repressed in cells overexpressing miR-125a compared to empty vector control cells. The functional role of many of these putative miR-125a targets specifically in HSCs remains to be determined. However, the two miR-125a predicted target genes that were most prominently affected, Glutaminase (Gls) and Ras-related protein Rap-1A (Rap1a), have been previously reported to affect HSC function. Gls is a K-type mitochondrial glutaminase expressed by different transcript variants mediated by alternate splicing. In HSCs, it has very recently been shown that the GLS protein is critical for the control of HSC self-renewal and that isoform switching of Gls is required for the activation of HSCs.¹⁶ Whereas increased

GLS levels maintain HSCs in a quiescent state, here we show that miR-125a decreases GLS transcripts, which coincides with increased HSC proliferation.

Table 1: MiR-125a predicted targets that are significantly downregulated in HSPCs upon miR-125a overexpression.

Gene	logFC	P-value	Function HSC	Function other
GLs	-5.60	2.41E-05	Required for activation ¹⁶	Glutamine metabolism ¹⁷
Rap1a	-4.98	5.38E-05	HSC maintenance ¹⁹	Cell adhesion, migration
Rab3d	-4.71	0.000116	Unknown	Intracellular vesicle transport ²⁰
Mfsd13a (TMEM180)	-4.24	1.79E-06	Unknown	Transmembrane protein ^{21,22}
Fam117a	-3.89	0.000416	Unknown	C/EBP-induced protein
Adams4	-3.75	0.000602	Unknown	Arthritis ²³
Nsmce3 (MAGEG1)	-3.38	0.000169	Unknown	DNA repair ²⁴
Ncf1	-3.30	1.89E-05	Unknown	NADPH oxidase component ²⁵
Ctsd	-3.01	4.86E-05	Lineage commitment ²⁶	Lysosomal protease
Nfe2	-2.86	0.000459	Erythroid/megakaryocytic lineage ²⁷	Transcription factor
Map11	-2.48	0.000538	Unknown	Associates with mitotic spindles ²⁸
Gjb2	-2.32	0.001171	Unknown	Gap junction protein

Gene: Gene name, alias in parenthesis. logFC: Fold change as determined by RNA-seq. P-value: P-value as calculated in RNA-seq. Function HSC: function in HSC or related to hematopoiesis. Function other: General function of protein.

Rap1a is a partner of Podocalyxin (Podxl) and can be activated by erythropoietin (Epo) or interleukin-3 (IL3). Rap1a is strongly activated when HSCs are challenged with granulocyte colony-stimulating factor (G-CSF) and can modulate cell migration and adhesion.^{17,18} Previously, we have established that miR-125a overexpressing HSCs respond more strongly to G-CSF compared to control cells.⁸ Other putative miR-125a targets that have previously been linked to lineage commitment, are the peptidase cathepsin D (Ctsd) and transcription factor Nuclear Factor, Erythroid 2 (Nfe2). The remaining transcripts that we identify as potential miR-125a targets in HSCs have no known function in the hematopoietic system thus far.

Molecular signatures that are associated with miR-125a-enhanced HSC potential

Beyond the potential identification of miR-125a targets, we further explored the overall molecular pathways that were affected by miR-125a overexpression. Although these changes may be caused by both direct or indirect effects of miR-125a, they will aid in better understanding of the phenotype that is observed in HSCs. We used the Molecular Signatures Database²⁹, to compare the 321 differentially expressed genes to different molecular signatures. Using this tool, we identified three molecular processes to be significantly enriched in this group of genes (Figure 7). The most strongly associated gene set contained transcripts involved in G2/M checkpoint control. This checkpoint prevents a cell from entering mitosis

in case of damaged DNA. Secondly, there was significant overlap between the differentially expressed genes upon miR-125a overexpression and targets of c-Myc, a transcription factor associated with metabolic changes and cell proliferation.³⁰ Lastly, we identified overlap with E2F targets that regulate cell cycle and DNA synthesis. These three most strongly associated gene sets are all involved in cell proliferation, which is in line with the observed functional and phenotypical changes of miR-125a overexpressing HSCs.

Figure 7: Top 3 enriched gene using all differentially expressed genes as input. The X-indicates whether a differentially expressed gene is present in one or more of the following sets: G2/M checkpoints³¹⁻³⁴ (G2M, $p=1.43E-12$), Myc targets³⁵⁻⁴⁴ (MYC, $p=1.66E-11$), and E2F targets⁴⁵⁻⁵³ (E2F, $p=1.79E-09$). Y-axis shows gene symbols, color codes indicate down- or upregulated genes in blue and red respectively. The size of each circle indicates the logFC.

DISCUSSION

In this chapter we show how continuous overexpression of miR-125a is required for the potent induction of HSC potential that is initiated by this miR, both in vitro and in vivo. This is in contrast to other examples of stem cell reprogramming, such as the induction of pluripotency in adult cells using Yamanaka factors, where a short expression leads to epigenetic changes that permanently alter the fate of the cell.^{54,55} Previous studies have associated long-term miR-125a overexpression with leukemia development.^{3,7} We show that HSCs that have been temporarily exposed to miR-125a overexpression do not show detrimental effects associated with excessive proliferation such as HSC exhaustion or leukemic derailment and still function normally in serial transplantation experiments. By temporarily overexpressing this miR, we show that it is possible to evoke the beneficial enhanced self-renewal capacity in HSPCs and at the same time prevent leukemia onset associated with chronic miR-125a overexpression.

In order to gain further molecular insight into the role of miR-125a and how it so potently induces HSC self-renewal, we established the transcriptome of cells in which mir-125a was overexpressed. Our current effort to identify potential miR-125a targets using RNA-sequencing complements various previous attempts to identify genes that must be repressed for miR-125a to exert its function in HSCs. These previous attempts have used targeted analysis, or unbiased microarray and mass spectrometry approaches. For example, targeted luciferase assays have indicated that Bak1 (BCL2 Antagonist/Killer 1) is a miR-125a target.⁵⁶ Another previously used approach is to filter micro array data for predicted targets obtained from miR-target database TargetScan.³ Since the involvement of a miR can alter protein expression of coding target genes, it has also been attempted to detect differential protein abundance. Mass spectrometry has identified Mitogen-Activated Protein Kinase 14 (MAPK14 or p38) and Protein Tyrosine Phosphatase Non-Receptor Type 1 (Ptpn1).⁷ We also previously identified Suppressor Of Cytokine Signaling 3 (Socs3) and Signal Transducer And Activator Of Transcription 3 (Stat3) and confirmed their differential expression after miR-125a overexpression by qPCR. We also showed that expression of c-Kit is repressed on HSPCs that overexpress miR-125.⁸ Collectively, a total of 6 studies have attempted to identify miR-125a targets in normal hematopoiesis, either by hand picking promising candidates or using higher throughput screening.^{3,4,7,8,57,58} Although a direct comparison of the results of these different studies is not straightforward due to the use of different experimental set-ups and models, it is remarkable that none of the various miR-125a targets appears to be consistently affected in these studies or in this current study, whereas there is a strong consensus on the functional changes that occur in HSCs upon miR-125a expression. The fact that little overlap is apparent if these various studies are compared suggests that miR-125a exerts its activity by relatively subtle repression of multiple targets.

We identify novel transcripts to be repressed upon miR-125a overexpression, including Glis which has recently been shown to be an important HSC regulator.¹⁶ We also show how differentially expressed RNAs upon miR-125a overexpression only partially overlap with predicted miR-125a targets. In addition, we identify multiple predicted miR-125a targets that are upregulated in HSCs and progenitor cells upon overexpression of miR-125a. As mentioned above, miR-125a alters the expression of the cKit receptor which we use to isolate HSCs. When isolating cells for RNA-seq, this marker could not be used to purify stem cells. Thus, we isolated a less primitive lineage Sca1⁺ population which also contains a large portion of progenitor cells. Therefore, the identified differentially expressed transcripts can be affected in both HSCs and progenitors or potentially only in one of the two populations. Upon overexpression of miR-125a the functional changes, such as enhanced proliferation, become immediately apparent. Our transcriptional analysis used cells isolated 12 weeks after initiation of the perturbation. Thus, the genetic perturbation and the resulting phenotypical changes that we report are likely the result of a complex interplay of both direct and indirect targets of the miR. Identifying direct targets of miR-125a will require additional studies that can confirm direct interaction between miR and mRNA sequence. Although it has proven to be challenging to identify critical miR-125a targets that are responsible for the induction of HSC self-renewal, continuous efforts to identify these targets are warranted to reveal the mechanisms behind the observed phenotype. This will potentially allow to harness our findings to enhance functionality and expansion of HSCs.

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CONFLICT OF INTEREST DISCLOSURE

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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SUPPLEMENTAL

Table S1: Differentially expressed genes top 100, sorted by P-value.

ensembl_gene_id	gene_name	logFC	logCPM	LR	PValue	FDR
ENSMUSG000000052415	Tchh	-8.6391	5.355879	40.89292	1.61E-10	2.74E-06
ENSMUSG000000029622	Arpc1b	10.17615	6.830706	34.40696	4.47E-09	3.81E-05
ENSMUSG000000068328	Aup1	7.326159	4.220552	33.42272	7.42E-09	3.90E-05
ENSMUSG000000034634	Ly6d	6.500173	5.598147	32.78114	1.03E-08	3.90E-05
ENSMUSG000000046805	Mpeg1	5.889052	5.06848	32.57695	1.15E-08	3.90E-05
ENSMUSG000000020267	Hint1	5.627275	4.821478	32.05087	1.50E-08	4.11E-05
ENSMUSG000000021250	Fos	5.641339	4.843191	31.82345	1.69E-08	4.11E-05
ENSMUSG000000056708	Ier5	6.966234	3.910042	30.06251	4.18E-08	8.91E-05
ENSMUSG000000028821	Syf2	7.958303	7.013904	29.06272	7.01E-08	0.000124
ENSMUSG000000105547	Iglc3	5.511907	5.504094	28.99225	7.27E-08	0.000124
ENSMUSG000000076940	Iglv2	7.960385	7.842875	27.95093	1.24E-07	0.000193
ENSMUSG000000051256	Jagn1	-7.29711	7.004021	27.61909	1.48E-07	0.000205
ENSMUSG000000047260	Emc6	4.75117	4.839847	27.51279	1.56E-07	0.000205
ENSMUSG000000031683	Lsm6	-4.53882	4.993548	27.00761	2.03E-07	0.000247
ENSMUSG000000076937	Iglc2	7.687892	8.782093	26.60331	2.50E-07	0.000284
ENSMUSG000000055839	Elob	-4.32389	4.783935	26.24643	3.01E-07	0.00032
ENSMUSG000000042312	S100a13	-6.22994	6.478225	26.02897	3.36E-07	0.000337
ENSMUSG000000107720	Gm43946	4.581244	5.140719	25.74035	3.91E-07	0.00037
ENSMUSG000000102905	Gm38266	-4.98782	5.721021	25.49513	4.44E-07	0.000398
ENSMUSG000000025580	Eif4a3	7.110426	7.571728	24.9315	5.94E-07	0.000506
ENSMUSG000000110834	Gm39469	4.349265	4.483434	24.62999	6.95E-07	0.000561
ENSMUSG000000019505	Ubb	5.833651	6.337674	24.54974	7.24E-07	0.000561
ENSMUSG000000069792	Wfdc17	4.690179	4.038947	24.37392	7.93E-07	0.000588
ENSMUSG000000033940	Brk1	-6.73476	7.416827	23.88954	1.02E-06	0.000724
ENSMUSG000000098542	Gm27532	-6.43137	3.477287	23.69791	1.13E-06	0.000768
ENSMUSG000000050666	Vstm4	-4.14478	4.185622	23.54594	1.22E-06	0.000786
ENSMUSG000000018433	Nol11	6.942534	9.157916	23.50463	1.25E-06	0.000786
ENSMUSG000000042712	Tceal9	5.156843	6.057245	23.33315	1.36E-06	0.000814
ENSMUSG000000056201	Cfl1	6.68652	7.780606	23.30172	1.38E-06	0.000814
ENSMUSG000000025227	Mfsd13a	-4.23714	5.367509	22.80557	1.79E-06	0.001018
ENSMUSG000000039813	Tbc1d2	-4.19992	5.341007	22.72758	1.87E-06	0.001026
ENSMUSG000000105601	Gm42725	-5.77921	6.692723	22.62905	1.96E-06	0.001046
ENSMUSG000000079477	Rab7	6.590729	8.544391	22.2906	2.34E-06	0.00121
ENSMUSG000000117022	Gm17920	-3.80215	4.349353	22.15489	2.52E-06	0.00126
ENSMUSG000000030978	Rrm1	6.528356	8.956281	21.67829	3.22E-06	0.00153
ENSMUSG000000073616	Cops9	3.805399	4.849583	21.67353	3.23E-06	0.00153
ENSMUSG000000056399	Prss34	-6.39708	8.683269	21.43588	3.66E-06	0.001685

Table S1: Differentially expressed genes top 100, sorted by P-value. (continued)

ensembl_gene_id	gene_name	logFC	logCPM	LR	PValue	FDR
ENSMUSG00000020570	Sypl	6.45985	8.948173	21.34579	3.83E-06	0.001719
ENSMUSG00000069662	Marcks	5.820486	7.011679	21.15591	4.23E-06	0.00185
ENSMUSG00000077309	n-R5s8	3.697928	4.74056	20.98736	4.62E-06	0.001969
ENSMUSG00000030654	Arl6ip1	6.346025	9.110899	20.9012	4.84E-06	0.002008
ENSMUSG00000044574	5031434C07Rik	-3.59363	4.529912	20.85711	4.95E-06	0.002008
ENSMUSG00000021018	Polr2h	-4.70785	6.155141	20.61629	5.61E-06	0.002191
ENSMUSG00000074039	4930520O04Rik	4.329629	3.762887	20.58108	5.72E-06	0.002191
ENSMUSG00000037152	Ndufc1	3.871368	4.102254	20.55796	5.79E-06	0.002191
ENSMUSG00000021248	Tmed10	-5.44032	6.845984	20.36524	6.40E-06	0.002368
ENSMUSG00000040747	Cd53	6.129505	8.571667	20.29051	6.65E-06	0.002368
ENSMUSG00000105057	Gm40190	-3.60401	4.178719	20.2855	6.67E-06	0.002368
ENSMUSG00000031400	G6pdx	5.03633	6.434973	20.2205	6.90E-06	0.002387
ENSMUSG00000027752	Exosc8	4.735422	6.183818	20.19177	7.01E-06	0.002387
ENSMUSG00000028609	Magoh	3.626732	4.355048	20.10864	7.32E-06	0.002405
ENSMUSG00000028271	Gtf2b	-5.34339	6.780008	20.10243	7.34E-06	0.002405
ENSMUSG00000073421	H2- Ab1	5.18896	6.610953	19.87605	8.26E-06	0.002655
ENSMUSG00000026385	Dbi	3.599732	4.967082	19.84135	8.41E-06	0.002655
ENSMUSG00000020612	Prkar1a	-5.88058	8.226044	19.61433	9.48E-06	0.002798
ENSMUSG00000038628	Polr3k	5.906337	8.262559	19.57564	9.67E-06	0.002798
ENSMUSG00000055095	Spink6	-3.40703	4.658243	19.53713	9.87E-06	0.002798
ENSMUSG00000014956	Ppp1cb	3.540032	4.929777	19.53032	9.90E-06	0.002798
ENSMUSG00000062937	Mtap	5.60156	7.230986	19.5012	1.01E-05	0.002798
ENSMUSG00000030966	Trim21	3.478492	4.838385	19.50036	1.01E-05	0.002798
ENSMUSG00000027306	Nusap1	5.901038	8.348211	19.48496	1.01E-05	0.002798
ENSMUSG00000024976	Shoc2	5.512023	7.119598	19.47713	1.02E-05	0.002798
ENSMUSG00000036594	H2- Aa	-5.87149	8.476797	19.29716	1.12E-05	0.002989
ENSMUSG00000002289	Angptl4	-5.31486	6.954562	19.2903	1.12E-05	0.002989
ENSMUSG00000031158	Timm17b	-4.6587	6.30775	19.22684	1.16E-05	0.003043
ENSMUSG00000060586	H2- Eb1	3.441996	4.807691	19.18333	1.19E-05	0.003066
ENSMUSG00000030045	Mrpl19	5.345546	7.016546	18.99943	1.31E-05	0.003312
ENSMUSG00000031358	Msl3	4.860342	6.487823	18.97889	1.32E-05	0.003312
ENSMUSG00000024098	Twsg1	3.599143	5.176464	18.89214	1.38E-05	0.003409
ENSMUSG00000033544	Angptl1	3.995496	5.711855	18.86865	1.40E-05	0.003409
ENSMUSG00000019942	Cdk1	5.450925	7.251382	18.76507	1.48E-05	0.003548
ENSMUSG00000061702	Tmem91	3.490736	5.067983	18.72385	1.51E-05	0.003575
ENSMUSG00000019795	Pcmt1	4.79954	6.471668	18.6912	1.54E-05	0.003587
ENSMUSG00000040767	Snrnp25	4.296022	6.030995	18.6367	1.58E-05	0.003612
ENSMUSG00000006127	Inpp5k	5.555839	7.504147	18.62645	1.59E-05	0.003612
ENSMUSG00000024845	Tmem134	3.445138	5.045258	18.56241	1.64E-05	0.003686

Table S1: Differentially expressed genes top 100, sorted by P-value. (continued)

ensembl_gene_id	gene_name	logFC	logCPM	LR	PValue	FDR
ENSMUSG000000055723	Rras2	-3.95582	5.798981	18.53087	1.67E-05	0.003699
ENSMUSG000000040242	Fgfr1op2	3.364215	4.500889	18.30791	1.88E-05	0.003978
ENSMUSG000000021748	Pdhb	5.213588	7.0496	18.3021	1.88E-05	0.003978
ENSMUSG000000015950	Ncf1	-3.28966	4.944598	18.29817	1.89E-05	0.003978
ENSMUSG000000014769	Psmb1	-5.50447	7.731819	18.29616	1.89E-05	0.003978
ENSMUSG000000020372	Rack1	5.44173	7.419811	18.20087	1.99E-05	0.004037
ENSMUSG000000024300	Myo1f	-5.10591	6.968488	18.18684	2.00E-05	0.004037
ENSMUSG000000042229	Rab1f	5.518029	7.886839	18.15663	2.03E-05	0.004037
ENSMUSG000000022969	Ili10rb	-5.32159	7.322472	18.15339	2.04E-05	0.004037
ENSMUSG000000004268	Emg1	5.074319	6.903899	18.13886	2.05E-05	0.004037
ENSMUSG000000104572	Fbxw23	-3.95811	5.853379	18.13194	2.06E-05	0.004037
ENSMUSG000000027133	Nop10	3.36024	4.991686	18.08405	2.11E-05	0.004062
ENSMUSG000000049327	Kmt5a	3.684462	5.47276	18.0331	2.17E-05	0.004062
ENSMUSG000000082440	Gm4914	-3.24905	4.253748	18.03047	2.17E-05	0.004062
ENSMUSG000000032475	Nck1	-5.59216	8.507545	18.0156	2.19E-05	0.004062
ENSMUSG000000086701	Gm13595	3.34169	4.954706	18.01356	2.19E-05	0.004062
ENSMUSG000000014232	Cluap1	5.214023	7.16671	17.96113	2.25E-05	0.004131
ENSMUSG000000116567	Gm49639	3.510382	5.272354	17.8607	2.38E-05	0.004291
ENSMUSG000000026103	Gls	-5.60266	8.752544	17.83543	2.41E-05	0.004291
ENSMUSG000000032053	Pou2af1	3.570782	5.363961	17.82832	2.42E-05	0.004291
ENSMUSG000000114851	Gm46409	-3.57732	5.496946	17.73976	2.53E-05	0.004445
ENSMUSG000000037058	Paip2	3.561335	5.368805	17.72209	2.56E-05	0.004445
ENSMUSG000000001403	Ube2c	4.517271	6.383617	17.69014	2.60E-05	0.004474

Table S2: Overlap analysis of both up and down regulated genes with hallmark gene sets from the Molecular Signatures Database at gsea-msigdb.org.Overlap UP+DOWN Hallmarks (<https://www.gsea-msigdb.org/>) 28-07-2020

Overlap Results

Collection(s):	H				
# overlaps shown:	10				
# genesets in collections:	50				
# genes in comparison (n):	321				
# genes in universe (N):	38404				
Gene Set Name	# Genes in Gene Set (K)	# Genes in Overlap (k)	k/K	p-value	FDR q-value
HALLMARK_G2M_CHECKPOINT	200	17	0.085	1.43E-12	7.17E-11
HALLMARK_MYC_TARGETS_V1	200	16	0.08	1.66E-11	4.14E-10
HALLMARK_E2F_TARGETS	200	14	0.07	1.79E-09	2.99E-08
HALLMARK_UV_RESPONSE_UP	158	10	0.0633	9.71E-07	1.12E-05
HALLMARK_OXIDATIVE_PHOSPH.	200	11	0.055	1.12E-06	1.12E-05
HALLMARK_MITOTIC_SPINDLE	199	10	0.0503	7.67E-06	4.46E-05
HALLMARK_GLYCOLYSIS	200	10	0.05	8.02E-06	4.46E-05
HALLMARK_HYPOXIA	200	10	0.05	8.02E-06	4.46E-05
HALLMARK_MTORC1_SIGNALING	200	10	0.05	8.02E-06	4.46E-05
HALLMARK_COMPLEMENT	200	9	0.045	5.19E-05	2.54E-04

Table S3: HSC staining protocol (LSK/SLAM) for primary and serial transplants.

LSK/SLAM staining

<i>antigen</i>	<i>color</i>	<i>clone</i>	<i>manufacturer</i>	<i>#</i>
Sca.1	Pacific Blue	D7	Biolegend	108120
cKit (CD117)	PE	2B8	Biolegend	105808
CD150	PE-Cy7	TC15-12F12.2	Biolegend	115914
CD48	A647	HM48-1	ITK	103416
B220	A700	RA3-6B2	Biolegend	103231
CD11b	A700	M1/70	Biolegend (ITK)	101222
CD3	A700	17A2	ITK	100216
Gr1 (Ly6G)	A700	RB6-8C5	ITK	108422
Ter119	A700	TER-119	ITK	116220

Staining		mix (μL)
Sca.1	Pacific Blue	45
cKit	PE	60
CD150	PE-Cy7	90
CD48	A647	45
B220	A700	18
CD11b	A700	90
CD3	A700	54
Gr1	A700	36
Ter119	A700	72

PI	-	-
GFP	-	-
mCherry	-	-

Total: 510

0.5μL/million cells after eryllysis, incubate 30 min. at 4C + wash

Table S4: Peripheral blood staining protocol.
Mature staining mCherry/GFP

<i>antigen</i>	<i>color</i>	<i>clone</i>	<i>manufacturer</i>	<i>#</i>	<i>lot</i>
CD45.2	BUV395	104	BD	564616	9099917
CD45.1	PE-Cy7	A20	Biolegend	110730	B137079
CD3	BV605	17A2	Biolegend	100237	B264993
B220	APC	RA3-6B2	Biolegend	103212	B114915
Gr-1 (Ly-6G/Ly-6C)	A700	RB6-8C5	Biolegend	108422	B258102
Mac-1 (CD11b)	A700	M1/70	Biolegend	101222	B308254
Brilliant stain buffer			BD	563794	6090696

Staining			dilute in (μL)	mix (μL)
CD45.2	BUV395	1	70	114.3
CD45.1	PE-Cy7	1	1400	5.7
CD3	BV605	1	400	20
B220	APC	1	800	20
Gr-1 (Ly-6G/Ly-6C)	A700	1	4000	2
Mac-1 (CD11b)	A700	1	4000	2
GFP	-	-	-	-
mCherry	-	-	-	-
DAPI	-	-	-	-
PBS+0.2% BSA + brilliant stain buffer			836	μL
Total:			1000	μL

10μL/sample after erylysis, incubate 30 min. at 4C + wash

Figure S1: Sorting strategy for bone marrow of primary recipients, gate marked in red dashed line indicates sorting gate. Hierarchy of gates according to alphabetical order of panels. (A) Cells, SSC-H vs FSC-H (B) Singlets, SSC-W vs FSC-H. (C) Living cells, PI vs. cKit-PE. (D) Lineage negative, lineage-A700 vs. CD48-A647. (E) GFP positive cells, GFP vs. cKit-PE. (F) Sorting gate LS, HSC gate LSK, cKit-PE vs. Sca1-PB. (G and H) HSC compartment LSK/SLAM of LSK compartment in G and of sorted LS compartment in H. CD48-A647 vs. CD150-PE-Cy7.

Figure S2: Sorting strategy of in-vitro expanded cells, gate marked in panel E with red dashed line indicates sorting gate for separation of GFP⁺ and mCherry⁺ cells. Hierarchy of gates according to alphabetical order of panels. (A) Cells, SSC-H vs FSC-H (B) Singlets, SSC-W vs FSC-H. (C) Living cells, PI vs. cKit-PE. (D) Lineage negative, lineage-A700 vs. CD48-A647. (E) GFP positive cells, GFP vs. cKit-PE. (F) Sorting gate LS, HSC gate LSK, cKit-PE vs. Sca1-PB. (G and H) HSC compartment LSK/SLAM of LSK compartment in G and of sorted LS compartment in H. CD48-A647 vs. CD150-PE-Cy7.

Figure S3: Engraftment of donor cells per mouse (n=5). Showing contribution of CD45.1⁺ donor cells to the recipient peripheral blood at 4-week intervals as a representation of donor cell engraftment. Each line represents consecutive sampling of the same recipient, each panel represents an experimental group. Experimental set up as shown in Figure 4 and 5.

4

An integrative approach to identify microRNA-125 targets in hematopoietic stem cells

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ABSTRACT

MicroRNAs (miRs) are small non-coding RNAs of ~22 nucleotides long. MiRs can regulate gene activity by inhibiting the translation of mRNA to protein. A single miR can have many different mRNA targets. The interaction between miR and target is mediated by the RNA-induced silencing complex (RISC). Pairing between miR and mRNA is based mainly, but not solely, on the 2-8 bases of the 5' end of a miR known as the seed sequence. In hematopoietic stem cells (HSCs), ectopic expression of any of the three miR-125 family members strongly enhances self-renewal. In order to better understand how miR-125 induces its potent phenotype, we aimed to identify miR-125 targets using various complementary approaches. Firstly, we performed RNA immunoprecipitation to specifically pull down the main component of the RISC, followed by RNA sequencing (AGO2-RIP-seq). We then integrated these newly acquired data with pre-existing experimental and database data. This led to the discovery of only a single gene, proteasome activator subunit 1 (Psmc1), highlighting the difficulty of reliably identifying miR targets using different unbiased screening techniques. Secondly, through a mutation analysis we identified the essential sequences of miR-125 required to induce hematopoietic cell proliferation. This led to the identification of a supplementary 3' interaction domain of miR-125 which we assume is essential for target recognition and/or repression.

Highlights

- Identification of microRNA-125 driven changes in RNA and protein abundance in hematopoietic cells
- Interaction between miR-125 and its target is not solely dependent on the 5' seed sequence
- Identification of a supplementary 3' target interaction domain of miR-125

INTRODUCTION

Hematopoietic stem cells are responsible for the replenishment of mature blood cells throughout life. The microRNA family 125 (miR-125) has an important role in the maintenance of hematopoietic stem cells (HSCs). The three family members of these small non coding RNAs are highly expressed in HSCs and expression declines upon differentiation.¹ When miR-125a is ectopically expressed in HSCs these cells acquire increased self-renewal potential.² In addition, enforced expression of miR125a in hematopoietic progenitor cells induces the potential to self-renew and provide multilineage engraftment after transplantation, which they normally lack. The ability to activate self-renewal programs in HSCs and progenitors will potentially allow for expansion of these rare cell populations. This can be of great clinical significance as the number of available stem cells is a limiting factor for treatment success.

MicroRNAs (miRs) are roughly 22 nucleotides long and can inhibit gene expression at the mRNA level. The mature miR is incorporated into the RNA-induced silencing complex (RISC) which mediates interaction between miR and mRNA. Upon overexpression of a miR, gene regulation is affected. First the miR interacts with its targets in the cytoplasm, which can lead to both stabilization or degradation of mRNA. In both cases the translation of the mRNA into protein is hampered, resulting in a difference of protein abundance. The seed region of a miR is thought to be mainly involved in the interaction of the miR with its mRNA target. The miR-125 family consists of three family members, 125a, 125b1 and 125b2, which all have the same seed region. In HSCs, the three miR-125 members have a similar role.³ For simplicity we use the name miR-125 when any of the three family members are implied.

In previous studies we have focused on the functional characterization of miR-125.¹⁻⁴ Additionally, several attempts have been made to uncover the mechanism by which this miR executes its remarkable effects. The first strategy applied was incorporation of stable-isotope labeled amino acids into cellular proteomes (SILAC), followed by mass-spectrometry (MS).² This allowed for the analysis of differentially expressed proteins in a murine hematopoietic cell line (32D), as well as human cord blood derived hematopoietic CD34⁺ cells. Secondly, we performed RNA-seq of murine HSCs overexpressing miR-125a.⁵ In the current study we performed RNA co-immunoprecipitation (AGO2-RIP-seq) of the same murine cell line, 32D, in which miR125a was overexpressed. This technique allows for the co-immunoprecipitation of the RISC complex and the mRNA it is interacting with, resulting in a pool of RNAs that is enriched for transcripts that are interacting with the RISC complex and the overexpressed miR-125a. Sequencing of the resulting RNA samples is expected to uncover the differentially pulled down transcripts upon miR-125a overexpression.

In addition to the global screening analysis, we adopted a more directed approach to unravel how miR-125 interacts with its targets. We first investigated the importance of the miR-125a 5' seed sequence. To this end, we functionally tested closely related miRs that had a seed-sequence or a 3' tail that was highly similar to the miR-125 family members. Secondly, we induced multiple point mutations in the miR-125b mature sequence and performed functional analysis in murine 32D cells and human cord blood derived CD34⁺ cells. This led to the identification of a secondary interaction domain in the 3' tail of miR-125.

METHODS

Cell lines - For in vitro experiments the murine bone marrow derived and IL-3 depended cell line 32D clone 3 was used. The cell line was maintained in RPMI medium supplemented with 10% FCS, 1% PenStrep and 100 ng/mL mIL3 (Preprotech).

Human cord blood derived CD34⁺ cells - Umbilical cord blood was collected from healthy donors at the Obstetrics Department of ISALA clinic in Zwolle, NL. PBMCs were isolated with the aid of SepMate tubes and Lymphoprep density gradient medium (STEMCELL Technologies). CD34⁺ cells were positively selected by magnetic separation using the a human CD34 MicroBead Kit and MACS columns (Milteny Biotech). Cells were frozen and stored at -150 °C in animal component-free CryoStor CS10 medium (STEMCELL Technologies).

Transduction - Viral supernatant was produced by transfecting the LentiX 293T packaging cell line (Clontech) using Eugene HD Transfection Reagent (Promega) according to manufacturer's protocol. Virus was harvested 48 hours after transfection and either used fresh or snap frozen and stored at -80 °C until use. Viral particles were bound to Retronectin (Clontech) coated plates according to the manufacturer's protocol. Cells were added to the virus-coated plates and spun down at room temperature at 400 g for 45 minutes to promote binding of the cells to the virus coated plate. Transduction efficiency was determined by flow cytometry using a FACS Canto II (BD Biosciences).

In vitro competition assays - Cells for the competition assay were transduced and maintained in culture conditions as described above. Transduced cells were stably marked with a fluorescent marker and mixed with wild type unmarked cells. Cells were maintained at the recommended density for optimal growth. Samples were taken twice per week, washed with PBS with 0.2% BSA and stained with DAPI. Marker contribution to the cell population were measured by flow cytometry and expressed as % marker chimerism of total living cells. Increasing chimerism indicating a proliferative phenotype for the marked cells, and inversely indicating a less advantageous phenotype upon decrease of the % marker chimerism.

AGO2-IP - In brief, 4×10^7 transduced 32D cells were harvested, washed in PBS and stored as a pellet at -80°C . Cells were lysed on ice in polysome lysis buffer with freshly added DTT, RNase inhibitor and protease inhibitors. For the IP procedure protein G Dynabeads (ThermoFisher) were used and coupled with AGO2 Monoclonal Antibody R.386.2 (ThermoFisher) in NT2 buffer for 1 hour at 4°C . After washing, the lysate was incubated with the beads for 2 hours at 4°C . During IP procedure both RNA and protein samples were collected from input, flow through and IP-fractions. Successful IP was determined by Western Blot. RNA was isolated with the MirNeasy mini or micro kit (Qiagen) according to manufacturer's protocol.

RIP-seq library preparation - RNA samples obtained from the IP procedure described above were checked for quantity, quality and purity by analysis on both Qubit (Invitrogen) and Bioanalyzer (Agilent). Library prep was performed with the SMARTer Stranded Total RNA-Seq Kit – Pico Input Mammalian (Clontech), according to the manufacturer's protocol. Total RNA input for cDNA synthesis was equalized based on the sample with lowest concentration. Fragmentation was performed for 4 minutes according to the obtained RIN values (>7). Library cDNA was pooled equimolarly and sequenced on the NextSeq 500 sequencing system (Illumina).

RIP-seq bioinformatic analysis - After demultiplexing, single unstranded reads were mapped to the murine genome (M13) using STAR aligner. After STAR-mapping the data were merged in the table of counts per gene (mouse genome GRCm38 version m13). Data consisted of 4 uniform replicates of: Control RIP, miR-125a RIP, and mutant-miR-125a RIP samples. After inspection, replicate 4 was rejected as it failed to meet quality control thresholds. The remaining 3 replicates showed substantial batch effects. Therefore, two protocols for batch compensations were applied. The first one was a BMC correction protocol (Mean Centered)⁶, written as custom python script. This script generally corrects to the mean average of every separate batch of data. In addition we used the well-known combat correction from the package 'sva'.⁷ The resulting reads data were converted to integers and used for 'Deseq2' protocol.⁸

Site directed mutagenesis - Mutations in miR-plasmids were introduced with primers designed using the NEBaseChanger (NEB) online tool. Primers were synthesized by IDT with 5' phosphorylation to allow for recircularization of the plasmid after amplification. Amplification was performed with Q5 polymerase (NEB) according to manufacturer's protocol. Successful insertion, deletion or substitution was confirmed with Sanger sequencing (GATC).

Plasmid amplification and purification - Plasmids were transformed into One Shot Top10 (NEB) competent *E. coli*. Colonies were grown on LB Agar (Invitrogen) or in LB Broth Base (Invitrogen) at 37°C, with 400 rpm shaking for liquid cultures. Plasmids were isolated from single clone liquid cultures using the GeneJET Miniprep kit (ThermoFisher) or Zymo-PURE™ Plasmid Midiprep Kit (Zymo).

RESULTS

AGO2-RIP-seq in murine hematopoietic cells to identify direct miR-125 targets

Direct interaction between miR and their target mRNAs occurs in the cytoplasm. This interaction is mediated by the RISC complex. The protein Argonaute-2 (AGO2) is an essential component of this complex. Immunoprecipitation of the RISC complex by targeting AGO2 allows for the co-precipitation of RNAs that are contained within the complex. For the purpose of identifying miR-125 targets in hematopoietic cells we first overexpressed miR-125a, an inactive 125a-mutant, and an empty vector control (EV) in the murine bone marrow derived cell line 32D. Overexpression was established by viral transduction, which also included a green fluorescent protein marker. GFP⁺ cells were sorted to enrich for cells in which integration of the vector had occurred, and immunoprecipitation was performed (Figure 1A). Successful precipitation of AGO2 was determined by western blot (Figure 1B). At the protein level we observe a low abundance of AGO2 in the input samples, we see no AGO2 in the flow-through indicating that most protein is bound to the beads and lastly, we see enrichment of AGO2 in the empty vector (EV-Ago) and miR-125a overexpression (125a-Ago) when IP is performed. At the same time the IgG isotype control does not show any aspecific pull down. Four replicates yielded RNA samples that were prepared for RNA-sequencing, one sample was later omitted as it did not pass quality control thresholds. (Figure 1C).

Prior to differential expression analysis, we validated the raw mapped RNA-seq reads to confirm our methods. Different to normal bulk RNA-seq, this experiment contained a specific pool of RNAs that are enriched for miR targets. Depending on the experimental condition this can also be additionally enriched for miR-125a targets upon overexpression of miR-125a. These factors make the analysis of the RNA-seq data non-standard and therefore require additional quality control steps. Figure 1D illustrates whether bio-informatically predicted miR-125a targets were present in the total pool of reads in a miR-125a overexpression sample. The three most abundant predicted targets are annotated with the gene name. This indicates that our most interesting transcripts are still present in the RNA sample and thus confirms our methods thus far. We performed an additional analysis, but this time checking for proteins that were previously found to be differentially expressed in the SILAC-

MS study² (Figure 1E). After we established that putative miR-125a targets we precipitated, we excluded overly abundant ribosomal RNA or mitochondrial RNA sequences (Figure S1 A-B). We then compared our data to another unrelated and independent AGO2-RIP-seq experiment (Geo Accession number: GSE109263)⁹ and concluded that our RNA-library was of comparable composition (Figure S1 C). Finally, we investigated the abundance of known AGO2 binders. Collectively, these analyses indicated that AGO2 pull-down experiment had been successfully performed.

Figure 1: AGO2-IP set up and validation. (A) Overall experimental set-up. (B) Validation of protein fractions at different stages of precipitation procedure. Input column represents total protein lysate used as input for IP. Flow-through shows protein flow-through in the washing steps. IP shows immunoprecipitated proteins. Rows are stained top to bottom with: AGO2, IgG or histone H3. (C) Overview of resulting RNA samples from pull down. Replicate number 'rep,' Sample ID 'id,' RNA concentration in the RNA sample in pg/ μ L and RIN quality score measured with the Bioanalyzer. (D-E) Overlap of raw mapped RIP-seq reads miR-125a targets and targets predicted by TargetScan (D) or targets previously identified using SILAC. (E) AGO2-RIP-seq data are represented by the blue line and red dots indicate overlapping genes with the other dataset. Y-axis indicates the average log2 of read counts, X-axis depicts sorted transcripts by rank (the most abundant genes are at the top left and the least abundant genes are at the bottom right). Top most abundant transcripts in the overlap are annotated with gene name.

Different genome-wide analysis methods reveal diverse and inconsistent miR-125a targets

We anticipated that the addition of the RIP-seq data set to already existing data would provide additional and complementary insight into genes targeted by miR-125a. The SILAC-MS dataset² shows changes that occur at the protein level, which can be either direct inhibitory effects of the miR, or rather changes that occur further downstream as a secondary result of the direct effects. The RNA-seq dataset⁵ shows which changes occur in mRNA levels, likely to be either influenced directly or indirectly by the presence of miR-125a. By immunoprecipitating the RISC complex by targeting AGO2, we expected to highly enrich the resulting RNA sample for direct miR-125a targets.

After the necessary validation steps shown in Figure 1, we performed differential expression analysis on the AGO2-RIP-seq data as explained in the methods, and we identified 573 differentially expressed genes. We then overlapped this list of differentially expressed genes with the two other available datasets (Figure 2A-B). Collectively, these three independent and complementary approaches were expected to result in at least partly overlapping target genes. Yet, when we searched for overlapping genes in these three independent datasets, overlap was minimal. In fact, we found only one single gene, proteasome (prosome, macropain) activator subunit 1 (Psmc1) to be consistently detected in all approaches (Figure 2A-B). Proteasomes are conserved protein complexes that eliminate proteins from a cell. Proteasomes exist in different subtypes, one of which is the immunoproteasome which has a key role in production of peptides needed in for antigen presentation.¹⁰ Psmc1 is an alternate regulator of the immunoproteasome, inducible by pro-inflammatory cytokine gamma-interferon. In HSCs, the proteasome is essential for normal function and loss of function can lead to leukemia.¹¹ In somatic stem cells the link between low protein synthesis and stem cell maintenance is well established. Recently, it has been shown that also HSCs synthesize less protein as compared to the more differentiated hematopoietic progenitors.¹² Accumulation of protein in the proteasome of HSCs impairs their function by inducing c-Myc expression, a regulator of HSC self-renewal.¹²

When searching for miR targets a number of bioinformatic databases are available that contain both predicted and experimentally validated targets. We utilized this available data and explored how many of the miR-125a targets that we experimentally had identified using RIP (present study), RNA-Seq (Luinenburg, Exp. Hematology) or MS (Wojtowicz, Cell Stem Cell) were present the publicly available databases. For all three experimental datasets we identified a small number of genes that were marked as miR-125a targets in both TargetScan¹³ and MirDB¹⁴ (Figure 2C). Utilizing the available information in these databases provides a tool to further investigate and narrow down lists of potential targets. Interestingly, none of the genes listed in Figure 2A was listed as a miR-125a target in the two

target databases mentioned before. Thus, if we integrate all available experimental data by simply overlapping, all predicted targets were eliminated. It is unclear whether this is due to the way we compare the datasets or flaws in target prediction algorithms.

Figure 2: Integration of three different genome wide analysis techniques. (A) List of genes that occur 2 or more times in all three experimental datasets. (B) Venn diagram of overlap between the three datasets. SILAC-MS², differentially expressed protein upon miR-125a overexpression in the 32D cell line. RNA-seq, differentially expressed genes upon miR-125a overexpression in primary hematopoietic stem and progenitor cells.⁵ RIP-seq: differentially expressed genes AGO2-RIP-seq data generated in this study. (C) Predicted miR-125 target genes by TargetScan and MirDB, and their overlap with RIP-seq, RNA-seq and SILAC-MS results respectively.

Interaction between miR-125 and its target is highly specific and not solely dependent on seed sequence

When comparing experimental data and predicted targets from databases, there was surprisingly little consensus on miR-125 targets. This may be partly explained by the way miR targets are bioinformatically predicted. For example, TargetScan uses the seed sequence of the miR and predicts targets by searching for conserved sites of 6-8 bases in targets. Additional TargetScan options also include other characteristics such as poorly conserved sites in the search and allowing for mismatches in the seed sequence. In order to improve target predictions for miR-125 it would be of great interest to know which exact bases are most critical for the biological effect to happen. Fortunately, overexpression of miR-125 results in a clear proliferative phenotype that can be quantified using a competition assay. In this assay we stably integrated a vector expressing the microRNA sequence, together with

fluorescent marker into the cells and allow these to proliferate in competition with wild type cells. When both populations proliferate at equal rates, the ratio of labeled and wild type cells will remain stable. Consequentially, when both populations proliferate at a different rate the ratio of labeled and wild type cells will change.

We previously observed that a single substitution in the middle of the seed sequence of miR-125a completely abolishes the phenotypical effects that are observed in HSCs upon miR-125a overexpression.³ This is indicative of the importance of the seed sequence in the interaction between miR-125 and its targets. It however does not exclude the involvement of other bases in the miR that are important for target binding. For all three miR-125 family members, the seed sequence is exactly the same. Small sequence differences in the tail of miR-125b distinguishes it from miR-125a (Figure 3A). We hypothesized that if the only important domain of miR-125 were the seed sequence, other miRs with the same seed sequence should have similar effects. To test this, we selected three naturally occurring miRs with a similar seed sequence (Fig 3A). We tested these alternative miRs in an in vitro competition assay in order to test whether these miRs were also able to initiate the proliferative phenotype similar to miR-125b. For this purpose, we used human cord blood derived CD34⁺ hematopoietic cells, allowing for additional in vitro testing such as colony forming potential of different hematopoietic lineage. For none of these alternative miRs we detected the proliferative phenotype that is induced when miR-125 is overexpressed (Fig3B-E). This indicates that the interaction between the miR and its targets in hematopoietic cells is not solely dependent on the seed sequence.

Additionally, we found that the effects of miR-125b could be inactivated in the same way as miR-125a, by substitution of the middle base of the seed sequence. This demonstrates that overexpression of the 125b mutant miR does not provide any proliferative advantage to cells compared to wild type.

Bases in the 5' end of the miR are involved in the interaction of miR-125 with its target mRNAs

The structure of human AGO2 protein allows for two interaction sites between miR and target RNA. First and most importantly, these sites involve the bases on position 2-8, the seed region. Secondly, there is supplementary binding in the 3' end of the miR that can increase target affinity more than 20 fold.¹⁵ In order to shed light on the mechanism by which miR-125 exerts its function we set out to further investigate supplementary binding sites in miR-125. A well-established strategy for investigating binding domain is to see which mutations cause disruption of binding. We used this strategy here to disrupt binding between the miR and its RNA target. For this purpose, we investigated several deletions and substitutions in both murine and human hematopoietic cells (Figure 4A). Caution should

Figure 3: Phenotype analysis of naturally occurring similar miRs. (A) Alignment of mature miR sequences (Clustal Omega 1.2.4). Bases in consensus with both miR-125a and miR-125a are highlighted in blue, seed sequence is boxed in red. (B-D) Competition assay of transduced human cord blood derived CD34⁺ cells. Reporter chimerism measured as %mOrange⁺ cells of total living cells in culture, increase of reporter chimerism shows proliferative advantage of transduced reporter positive cells over the untransduced wild type cells. (C-E) Increase of %mOrange⁺ between first measurement (day 2) and final measurement (day19/20).

be exercised when designing miR mutants as immature miRs form hairpin structures, and disruption of these secondary structures will hamper proper nuclear export and maturation. Since our overexpression method stably integrates the miR-125 gene in its genomic context, activity still relies on the ability of the cell to properly express miRs. The selected mutant miRs did not have major disruption of the predicted hairpin structures (Figure S2).

In a competition assay we again assessed the effects of the mutated miRs on cell proliferation. We compared the effects of the various mutant constructs with wild type (WT) miR-125b and the empty vector that had no proliferative advantage over the non-labeled cells

in the culture. In Figure 4B this shows as a highly proliferative phenotype that dominates the culture after 30 days (125b) or stable chimerism around 50% of the culture (EV). In murine cells we observed how the deletion of the G at position 19 (del19G), caused an intermediate phenotype. These cells showed a proliferative advantage over the empty vector control cells (EV), but the effect was smaller than for WT miR-125b. Reversely, deletion of the first T (del1T) had a negative effect on cell proliferation. Deletion of both deleterious 1T

Figure 4: Phenotype analysis of miR-125 mutants. (A) Alignment of mature miR sequences (Clustal Omega 1.2.4). Bases in consensus with both miR-125a and miR-125a are highlighted in blue, seed sequence is boxed in red. Mutations are marked with a pink asterisk. (B) Competition assay upon overexpression of miR-125b mutants. In murine hematopoietic cell line 32D. (C) Delta of reporter chimerism between start and end of competition assay. Where >0 = increased proliferation, <0 = decreased proliferation. (D) Competition assay in human cord blood derived $CD34^+$ cells. (E) Colony forming unit (CFU) assay in $CD34^+$ cells from panel D. CFU-GEMM = granulocyte, erythrocyte, monocyte, megakaryocyte; CFU-GM = granulocyte, monocyte; CFU-M = monocyte; BFU-E = burst forming unit erythroid.

and beneficial 19G (DM) neutralized the effect on cell growth. Interestingly, substitution of the 16th (16G) or 19th (19C) base caused cell death, but only in the murine cells. The growth dynamics of the different conditions are difficult to compare due to the internal competition with non-labeled cells. We therefore represent the differences in growth as deviation from the baseline chimerism at day 0, i.e., proliferative advantage shows as a positive value with a maximum of 50 and proliferative disadvantage shows as a negative value with a minimum of -50. (Figure 4C) We detected statistically significant differences between EV (4.8 ± 1.6) and WT miR-125b (45.7 ± 3.3 , $p = 0.0004$), EV vs. miR-125b del1T (-24.9 ± 1.9 , $p = 0.003$) and EV vs. miR-125b del19G (22.1 ± 3.6 , $p = 0.01$).

A preliminary test of these same miR mutations in human cord blood derived CD34⁺ cells, showed a similar pattern for the double mutant (DM), which neutralized the effect of miR-125 on the cells. Both single deletions (del1T, del19G) showed an intermediate phenotype. The substitutions that caused cell death in the murine cells (sub16G, sub19C), did not show this in the human cells (Figure 4D). An important characteristic of the phenotype that was induced by miR-125 is that proliferation of HSCs did not cause stem cell exhaustion. Upon overexpression of the different mutant miRs in human CD34⁺ cells we observed that the mutations provided a proliferative rate above the baseline of control cells (all except del19G) also ensured colony forming unit ability of the most primitive colonies as compared to miR-125b (Figure 4E).

DISCUSSION

In this chapter we set out to further define targets of miR-125. First, we performed AGO2-RIP-seq to identify miR-125 targets in murine hematopoietic cells. We then compared these results with previously performed unbiased screening for miR-125a targets on RNA⁵ (Chapter 3) and protein level.² From this comparison it was clear that the various identified miR-125a targets showed little consistency between different screening techniques. Second, we further investigated the interaction domain of miR-125. Interaction between miR-125 and its targets through the 5' 2-8 bases known as the seed was already confirmed previously.³ Substitution of the middle base of the seed sequence (T>C on position 4) leads to a complete reversal of the proliferative phenotype that is observed upon miR-125 overexpression. Moreover, we show that other miRs with similar seed sequences to miR-125 are not able to instigate the same effects on cell proliferation, indicating that the seed sequence is not solely responsible for the interaction between miR and target leading to the phenotypical changes that are observed in HSCs. Using mutation studies, we identified an additional region in the 3' end of miR-125 that is, in part, responsible for the efficient interaction with targets.

The data from the previously performed SILAC-MS and RNA-seq experiments and the newly generated AGO2-RIP-seq provides snapshots of miR-125a effects on different regulatory levels. The datasets give insight in the changes that occur in protein expression, RNA expression, and miR-RNA interaction respectively. It is important to keep in mind that these processes are dynamic and continuously interacting. In order to gain a better understanding on the molecular processes that change inside the cell upon miR-125a overexpression we aimed to integrate these different data sets. An overlap analysis of the differentially expressed genes yielded only one consistent gene between the three datasets: proteasome (prosome, macropain) activator subunit 1 (Psme1). A major challenge in these experiments is the ability to differentiate between direct and indirect effects on expression. In this case a more targeted yet still unbiased screening approach for miR targets would be preferable. In this study we therefore performed AGO2-RIP-seq. This technique is similar to RNA-seq but with a pool of RNA that is enriched for potential miR-125a targets. A useful improvement of this method would be to also crosslink miR and RNA with the use of ultraviolet light. This method is known as CLIP-seq¹⁶ and allows for the analysis of physical interaction between the two RNA molecules. Combining CLIP-seq with RNA-seq has already shown to improve target predictions.¹⁷

Current technological advances make large unbiased screening approaches such as RNA-seq more widely available. A large amount of data can be generated giving broad insights into cell expression changes. In the case of studying miR mechanisms with the goal of identifying targets, this has shown to be challenging, although progress is made.^{18–20} Different screening methods yield different and inconsistent target lists. Part of the challenge originates from the mechanism by which a miR can have its effects on a cell. Targeting of a mRNA will not always lead to degradation of the mRNA, making it impossible to find this effect when performing normal RNA-seq. At the same time, a cell can mitigate the effects of a miR by upregulating the targeted mRNA, causing the down-stream effect on protein expression to disappear. On top of this it is important to keep in mind that substantial differences can also occur in different cell types. When expression levels of miR targets change, the effects that the miR can have on these mRNAs also changes. A target that is strongly affected in hematopoietic stem cells might not be expressed at all in other tissues, and vice versa.

Although the magnitude of data generated by large screening approaches can be very tempting, investigating the interaction between miR and target might in the end be more insightful on a micro scale.

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CONFLICT OF INTEREST DISCLOSURE

No financial interest/relationships with financial interest relating to the topic of this chapter have been declared.

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SUPPLEMENTARY

Figure S1: AGO2-RIP-seq quality control.

Figure S2: Hairpin structures of miR-125 mutants. Red lines indicate deletions, red arrows indicate substitutions.

5

MicroRNA-125 to expand human cord blood-derived CD34⁺ hematopoietic stem and progenitor cells

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ABSTRACT

Human umbilical cord derived CD34⁺ cells are an easily accessible source of hematopoietic stem cells (HSCs). Lifesaving transplants have been performed using these HSCs. A major drawback however is the limited number of cells that can be obtained from a single umbilical cord, which is often insufficient for the successful transplantation of an adult patient. An alternative source of transplantable HSCs could come from induced pluripotent stem cells (iPSCs). Advances in this field show promising results when attempting to generate HSCs from iPSCs. MicroRNA-125 (miR-125) has shown to be a potent enhancer of HSC expansion both in vitro and in vivo. In this study we show a fold expansion of ~120 of human cord blood derived CD34⁺ cells in which miR-125 is overexpressed. We also attempted to increase the potency of iPSC derived CD34⁺ cells by overexpressing miR-125a. Lastly, we show that the expression of miR-125a must be continuously present in order to govern its effects. Together, these findings show the potential of miR-125 as a tool for the efficient in vitro expansion of HSCs.

Highlights

- Expression levels of miR-125a needs to exceed a threshold to have phenotypical effects in HSCs
- MiR-125 can expand human CD34⁺ cells ~120 fold in vivo
- Continuous expression of miR-125a is required to induce human HSC expansion

INTRODUCTION

Human cord blood derived CD34⁺ cells are an easily accessible source of hematopoietic stem and progenitor cells (HSPCs). These HSPCs can be efficiently isolated from the blood that remains in the umbilical cord and placenta after delivery.¹ Cord blood derived hematopoietic stem cells (HSCs) can be used to treat both children and adults with bone marrow insufficiency resulting from hematological malignancies or other inherited hematological diseases.^{2,3} Especially when a Human Leucocyte Antigen (HLA) compatible related or unrelated donor is not available, cord blood derived HSCs can be a suitable alternative. However, the number of HSCs that can be obtained from a single cord blood sample is limited and highly variable, and strict selection criteria must be met to maximize successful engraftment.⁴ Methods that can expand HSCs can be of great clinical significance. Increasing the stem cell dose in an HSC transplantation increases the chance of successful engraftment and reconstitution of the hematopoietic system.^{5,6}

MicroRNAs (miRs) are small non-coding RNAs of ~22 nucleotides long that can influence gene expression post-transcriptionally.⁷ A single miR has many different mRNA targets. MicroRNA-125 (miR-125) is a family of three highly evolutionary conserved miRs, miR-125a, miR-125b1 and miR-125b2. All three family members of this miR family enhance HSC function.⁸ Moreover, the overexpression of miR-125a induces long-term repopulating capacity in hematopoietic progenitors, an ability they normally lack.⁹ These properties make miR-125 a promising tool for enhancement and expansion of HSCs.

Two different approaches can be considered to reach the goal of increasing the number of available hematopoietic stem cells. First, it may be possible to expand existing sources of HSCs such as mobilized adult HSCs or HSCs from umbilical cord blood. A major technical challenge is the *in vitro* culture of these cells. HSCs require specific culture conditions that prevent terminal differentiation of these multipotent cells, a feature which is very commonly seen in *ex vivo* expansion protocols. Excitingly, recent methodological advances have greatly improved these *ex vivo* culture protocols.¹⁰ A second approach is to generate HSCs from other, non-hematopoietic, sources such as induced pluripotent stem cells (iPSCs). iPSCs can be derived from mature cells that are reprogrammed with transcription factors that induce pluripotency in any adult somatic cell.^{11,12} It has been shown that it is possible to use iPSCs and differentiate these *in vitro* to a hematopoietic stem cell like phenotype.^{13,14} However, the multilineage engraftment potential of transplanted iPSC-derived HSCs has been minimal.¹⁵

In this chapter we investigate whether enforcing the expression of miR-125 in *ex vivo* cultured HSCs leads to stem cell expansion. We also explore whether miR-125 can induce the engraftment potential of iPSC-derived HSCs. Finally, we utilize our previous findings

on the reversibility of miR-125a expression in murine HSCs¹⁶, and investigate the effects of reversal of miR-125a expression back to endogenous levels in human cord blood derived CD34⁺ cells.

METHODS

Mice - NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ immune deficient mice (NSG) were bred in the Central Animal Facility of the University Medical Center Groningen. All animal experiments were approved and performed according to guidelines of the University of Groningen Animal Care Committee.

Isolation of CD34⁺ HSCs from umbilical cord blood - Umbilical cord blood was collected from healthy donors at the Obstetrics Department of ISALA clinic in Zwolle, NL. PBMCs were isolated using SepMate tubes and Lymphoprep density gradient medium (STEMCELL Technologies). CD34⁺ cells were positively selected by magnetic separation using the human CD34 MicroBead Kit and MACS columns (Milty Biotech). Cells were frozen and stored at -150 °C in animal component-free CryoStor CS10 (STEMCELL Technologies).

Transduction - Cord blood derived CD34⁺ cells were thawed and incubated for 24 hours at 37 °C in StemSpan medium supplemented with 100 ng/mL TPO, SCF and Flt3-L. Viral particles were produced in various packaging cell lines. Retrovirus was produced in a combination Platinum-Eco and PG13 cell lines, or directly in the amphotropic envelope producing Platinum-Ampho. Lentivirus was produced in the LentiX 293T packaging cell line (Clontech). All packaging lines were transfected using FuGENE HD transfection reagent (Promega). Transduction was performed by pre-binding the virus particles to RetroNectin-coated (TakaraBio Inc.) plates in line with the manufacturer's protocol. In brief, virus was pre-bound to the plate by spinning at 1000g for 90 minutes after which they were placed back in the incubator for a minimum of 2 hours. After incubation virus was removed from the wells, washed once with PBS, cells added in fresh media and spininfected at 400g for 45 minutes.

Plasmids - The MXW-pPGK-IRES-eGFP retroviral vector used for overexpression of miR-125a was constructed as previously described.^{8,17} The lentiviral vector for miR overexpression was also used as previously described.¹⁸ The reversible lentiviral vector was created by combining the previously described reversible retroviral expression plasmid¹⁶ and the lentiviral vector for miR expression. The LoxP cassette was amplified from the retroviral vector using forward primer: taagcaGAATTCcctgcactcgagataacttcg and reverse primer: taagcaACCGGTgcaggtcgactactgtacag. The amplified product was inserted into the retroviral backbone by using the EcoRI and AgeI restriction sites. Resulting in a lentiviral

plasmid with a reversible miR-125a overexpression cassette, with GFP fluorescent marker upon overexpression and mCherry fluorescent marker upon reversal.

Bone marrow transplantation - Female NSG mice, 2-6 months of age, were used for xenograft studies. Mice were sublethally irradiated with 1.8 Gy 4-6 hours prior to transplantation. Cells were injected retro-orbitally, mice did not receive any supporting cells or antibiotics. Efficiency of transduction was measured in vitro at 4-6 days after transplant by measuring the fluorescent marker expression by flow cytometry (Canto II, BD Biosciences). Peripheral blood samples were taken every 4 weeks starting from week 6 post-transplant.

Analysis of peripheral blood - Whole blood was obtained by orbital puncture, ~60µL per sampling. 2µL whole blood was used for erythrocyte and platelet analysis. Erythrocytes were stained at room temperature with CD41a-PECy7 (#561424, BD Biosciences) and CD235a-APC (#561775, BD Biosciences) in PBS+0.2% BSA for 30 minutes. Erythrocyte lysis buffer was added to the remaining blood sample. White blood cells were stained with CD45-APC (#304016, Biolegend), CD33-BV421 (#562854, BD Biosciences), CD3-Apc-Cy7 (#561800, BD Biosciences) and CD19-FITC (#561741, BD Biosciences), in PBS+0.2% BSA for 30 minutes at 4°C.

In vitro assays - In vitro stem cell and progenitor frequency assays were performed with CD34⁺ cord blood derived cells transduced and isolated as described above. CFU assays were performed using Methocult H4435 (STEMCELL Technologies) according to manufacturer's protocol. CAFC and LTC-IC assays were performed on an MS5 feeder layer in Myelocult H5100 (STEMCELL Technologies) supplemented with 10⁻⁶ M Hydrocortisone according with weekly 50% medium changes according to the manufacturer's protocol, with exception of the LTC-IC step where the cells were not reseeded in Methocult but instead the assay was performed directly in the 96-well plate by replacing the 200µL Myelocult for 50µL Methocult. CAFC scoring was performed after 5 weeks of culture, LTC-IC was initiated at this point by the addition of Myelocult and scored at week 7.

Induced pluripotent stem cells - iPSC-derived CD34⁺ cells were provided by Marten Hansen at Sanquin, Amsterdam. These cells were generated from human iPS cell lines¹⁹ using an established hematopoietic differentiation protocol¹⁴. In brief: iPS colonies were differentiated in Stemline II supplemented with 1% Pen/strep, 50 ng/ml bFGF, 40 ng/ml VEGF, 20 ng/ml BMP4 and 100× insulin-transferrin-selenium. At day 6 the colonies were harvested and CD34⁺ cells were positively selected by magnetic separation using the human CD34 MicroBead Kit and MACS columns (Milteny Biotech). After arrival in our facility, the cells were transduced as described above with the exception that these cells were spinfected at 200g with medium brake.

RESULTS

High levels of miR-125 are required to expand HSCs

To overexpress miR-125 we virally introduced the miR gene with a fluorescent label to CD34⁺ cord blood-derived cells by transduction. This method allows for stable integration whilst at the same time fluorescently labeling the cell and all its progeny. Different properties of a viral vector can influence the expression that is achieved in the cell of interest. In this experiment we overexpressed miR-125 in cord blood derived CD34⁺ and characterized the cells both in vitro and in vivo. (Figure 1A) We tested two different vectors, a retroviral vector (*MXW-pPGK-IRES-eGFP*) that was previously used in murine cells only⁸ and a lentiviral vector (*SFFV-EF1a-mOrange-WPRE*) that was previously used to overexpress miR-125a in human cells.⁹ (Figure 1B) Upon transduction the retroviral vector induced an average fold change of 3.7 for miR-125a and 1.7 for miR-125b in cord blood derived CD34⁺ cells. In contrast, the lentiviral vector induced a fold-change of 232.3 for miR-125a and 134.8 for miR-125b. (Figure 1C) When tested in an in vitro competition assay, only the lentiviral vector caused the cells to show the proliferative phenotype that is associated with miR-125 overexpression. (Figure 1D) In this assay, cord blood derived CD34⁺ cells were transduced and maintained in vitro for 10 days. The initial measurement of reporter chimerism at day 0 represents the transduction efficiency, which was between 20 and 40 percent. The remaining cells in the culture serve as untransduced control cells. For each condition the percentage of reporter positive cells was measured at regular intervals. When transduced cells acquire proliferative advantage compared to their untransduced counterparts, the percentage of reporter positive cells increased. When this was not the case, the percentage of reporter positive cells remained stable. When we transplanted HSCs from both groups and their respective controls in immune-deficient recipient mice, only the lentiviral vector increased miR-125 expression enough to activate the increased HSC potential that is associated with ectopic expression of miR-125. (Figure 1E-F) HSCs that were transduced with the retroviral plasmid did not show increased contribution to the peripheral blood compared to the empty vector controls (Figure 1E) Engraftment of human cells was clearly visible in the peripheral blood of the mice transplanted with both the retrovirally and lentivirally transduced cells. (Figure 1G-H) Indicating that the engraftment of human cells into the murine host was successful, but initiation of miR-125a mediated expansion was not achieved, likely due to the low overexpression levels of the retroviral vector. These data indicate that the overexpression of miR-125 needs to exceed a threshold in order to induce HSC proliferation.

Enforcing miR-125a expression results in expansion of human cord blood derived CD34⁺ cells

To assess the potential of miR-125a to expand human cord blood derived HSC we set out to further investigate the expansion kinetics in the following experiment. Similar to the previ-

Figure 1: Overexpression of miR-125a needs to reach a threshold in order to govern its effects on HSCs. (A) Experimental setup. (B) Overview of expression vectors used. Top: previously used retroviral vector, MXW-pPGK-IRES-eGFP. Bottom: lentiviral vector more suitable for miR overexpression in human cells, SFFV-EF1a-mOrange-WPRE. (C) Expression fold change of miR-125a/b by transduction with retroviral or lentiviral vector. Values were normalized to empty vector control, relative to 2 control miRs (miR-16 & miR-191). (D) Competition assay of human cord blood derived CD34⁺ cells upon overexpression of miR-125a with the two different expression plasmids. Cells were maintained in vitro for 10 days in competition with untransduced wild type cells. Proliferative advantage shows as an increase of reporter chimerism over time (only lenti-125a condition), normal proliferation shows a stable percentage over time. (EV conditions and retro-125a condition) (E-F) Reporter positive cells in peripheral blood of recipient mice, retroviral plasmid in panel E, lentiviral plasmid in F. Week = weeks post-transplant and %GFP⁺/mOrange⁺ = reporter positive cells as a percentage of the total white blood cells in the peripheral blood. An increase of reporter percentage over time indicates expansion of the transduced HSCs. (G-H) Contribution of donor cells (hCD45⁺) to peripheral blood in recipient mice. Week = week post-transplant, %hCD45⁺ = percentage of human CD45⁺ cells in the peripheral blood.

ous experiments, we transduced cord blood-derived CD34⁺ cells and performed in vitro competition assays and a xenograft transplant into sub-lethally irradiated immunodeficient NSG mice. (Figure 2A) In vitro we observed enhanced proliferation of CD34⁺ cells when overexpressing both miR-125a and miR-125b. (Figure 2B-C) In order to assess the expansion potential of human CD34⁺ cells in vivo we performed a transplantation of human HSCs into a murine immunodeficient recipient. We transplanted NSG mice with human HSCs overexpressing miR-125a or miR-125b, and used an empty vector control and a functionally inactive miR-125a mutant previously described.⁸ Ten-twelve weeks post transplantation we observed an increased percentage of human CD45⁺ (hCD45⁺) cells in the bone marrow upon overexpression of miR-125a or miR-125b. (Figure 2D) Additionally, we investigated the distribution of transplanted cells in two different anatomical locations, the spine and the legs (consisting of femur, tibia and the pelvic bone). Both anatomical locations contain a comparable percentage of transplanted cells, indicating that the cells distribute evenly over the different locations. In the bone marrow we also observed an increased percentage of CD34⁺ HSPCs upon overexpression of miR-125a or miR-125b. (Figure 2E)

In order to quantify the magnitude of expansion of CD34⁺ cells, we first estimated the total number of CD34⁺ cells present in the bone marrow. This number was calculated by multiplying the total nucleated cell count in the bone marrow sample with the CD34⁺ percentage measured by flow cytometry. (Figure 2F) This representation only showed a marginal difference between the experimental groups. These numbers however do not take into account the difference in number of transplanted (stem) cells. While the total number of transplanted cells is controlled, the number of transduced cells that are present in the sample differs as a consequence of differences in transduction efficiency. In these experiments, the transduction efficiency was higher in the empty vector and seed-sequence mutant controls. The corrected, transduced, cell doses for the different groups were: miR-125a: 0.22×10^6 , miR-125b: 0.24×10^6 , empty vector: 0.93×10^6 , seed-sequence mutant: 0.86×10^6 . When we corrected for this difference in transplantation dose, we observed a clear increase in the expansion of CD34⁺ cells upon both miR-125a and miR-125b overexpression. While in control conditions the cells expanded in vivo ~22 (empty vector) to 24 (seed-sequence mutant) fold, both miR-125a and miR125b overexpressing cells expanded ~125 fold. (Figure 2G)

In previous murine transplantation studies we observed myeloid skewing and leukemia upon miR-125 overexpression.^{8,9} Upon overexpression of miR-125a, murine HSCs showed an increased contribution to the myeloid lineage. In many cases the increased proliferation developed into a leukemia, leading to decreased survival in the mice transplanted with miR-125a overexpressing HSCs compared to empty vector controls. In this experiment we did not observe this effect in human HSCs overexpressing miR-125a or miR-125b. (Figure 2H) In the bone marrow of mice transplanted with human HSCs overexpressing miR-125a or

Figure 2: MiR-125a can be used as a tool for the expansion of human cord blood derived CD34⁺ cells. (A) Experimental setup. (B-C) Expansion of CD34⁺ cells upon overexpression of miR-125a (B, n=1) or miR-125b (D, n=2). (D) Percentage of hCD45⁺ cells in the bone marrow (BM) of recipient mice. Statistically significant difference tested with 2way ANOVA, EV vs. 125a, p=0.0078; EV vs. 125a p=0.0207. (E) Percentage of CD34⁺ cells in the bone marrow (BM) of recipient mice. Statistically significant difference tested with 2way ANOVA, EV vs. 125a, p=0.0012; EV vs. 125a p=0.0103. (F) Total number of CD34⁺ cells present in bone marrow of recipient mice. (G) Fold change expansion of CD34⁺ cells, total number of CD34⁺ cells from panel F corrected for transplantation dose. Transplant doses (in millions): 125a: 0.22, 125b: 0.24, EV: 0.93, MUT: 0.86. (H) Lineage distribution in bone marrow, total number of cells. Gran = Granulocytes, Myelo = Myeloblasts, B = B-cells, T = T-cells.

miR-125b we did observe a higher total number of cells. The lineage distribution, however, is not significantly different between miR-125a and miR-125b compared to the empty vector and seed-sequence mutant controls.

Mir-125a as tool to induce engraftment potential in iPSC derived CD34⁺ cells

We hypothesized that overexpression of miR-125a might also enhance the engraftment potential of iPSC derived CD34⁺ cells. To investigate this, we transduced iPSC-derived CD34⁺ cells with the lentiviral vector to overexpress miR-125a and transplanted transduced unsorted cells into sublethally irradiated NSG mice. (Figure 3A) The iPSC-derived cells were more variable in cell size, granularity and CD34 expression when compared to cord blood derived HSCs. (Figure 3B) Our normal transduction protocol yielded high transduction efficiencies of the iPSC-derived cells. (Figure 3C) Unfortunately, upon transplantation none of the recipients of the iPSC-CD34⁺ transplant showed any contribution to the peripheral blood in leukocytes (Figure 3D), erythrocytes or platelets. At the same time, the cord blood-derived control sample showed a steady increase in reporter positive cells in the peripheral blood. The bone marrow of these recipients was analyzed 18 weeks post-transplant. In the bone marrow, or any of the secondary locations for hematopoiesis, such as spleen and liver, no iPSC-derived cells were detected. Since the iPSCs that were used for this experiment were initially derived from megakaryocytes we also evaluated presence of donor cells in the lungs, a potential megakaryocyte niche²⁰, for any marker positive cells. Again, no progeny of the iPSC derived cells was identified, showing that these cells were not able to engraft into the recipient.

Continuous expression of miR-125a is required to induce human hematopoietic stem cell expansion

In previous work we established that continuous expression of miR-125a is required to induce murine hematopoietic stem cell repopulating activity.¹⁶ In order to investigate this same phenomenon in human hematopoietic cells we adapted the reversible expression plasmid in order to optimize miR expression in human cells. To this end we subcloned the Cre-LoxP cassette from previous work into the lentiviral backbone that was used for the other experiments in this study. This allowed for the Cre-mediated reversible expression of miR-125a in cord blood-derived CD34⁺ cells. In a pilot experiment human HSPCs were transduced, cultured and after three days the overexpression of miR-125a was reverted to endogenous levels. (Figure 4A) The Cre-mediated reversal also switches the fluorescent label of reversed cells from the green fluorescent protein GFP to the red fluorescent protein mCherry. (Figure 4B) This allows for tracking of both overexpressing and reversed cells by flow cytometry. We assessed the following parameters in the in vitro culture: cell viability, marker chimerism, CD34⁺ percentage and the more primitive CD34⁺CD38⁻ percentage.

Figure 3: Transplantations with iPSC derived CD34⁺ cells. (A) Experimental setup. (B) Flow cytometric analysis of CD34⁺ cells derived from cord blood (pink) or iPSCs (blue). Left: SSC-A vs. FSC-A plot indicating cell granularity and cell size. Right: CD34-APC vs. FSC-A plot showing CD34⁺ cell populations. (C) Cytospot of transplanted cells, Giemsa stain. Top: Cord blood derived CD34⁺ cells, bottom: iPSC-derived CD34⁺ cells. (D) Transduction efficiency of experimental conditions, measured 48 hours after transduction. (E) Contribution of human CD45⁺ cells to the peripheral blood of the transplanted mice. (n=4) (F) Percentage of mOrange⁺ miR-125a overexpressing WBCs in peripheral blood of transplanted mice.

(Figure 4C) None of these parameters revealed major differences between miR-125a overexpression and empty vector control, and also no differences between overexpressing (GFP⁺) and reversed (mCherry⁺) cells were detected.

In addition to the measurements of different variables by flow cytometry (viability, marker chimerism, CD34 and CD38 expression), cells were manually counted at each passage. This resulted in a growth rate between two time points that can be extrapolated to a total number of cells, as if the cells were allowed to grow freely without the restricted volume of media in a culture condition. The total number of cells was then corrected with the flow cytometry parameter of interest, estimating the total amount of live CD34⁺ and CD34⁺CD38⁺ cells.

In all cases cells that overexpress miR-125a outperformed control cells, with a higher number of viable, CD34⁺ and CD34⁺CD38⁻ cells (Figure 4D). Compared to the empty vector controls, miR-125a overexpressing cultures contained respectively 1.8, 1.5, 1.6 and 2.4 times more live, reporter positive, CD34⁺ and CD34⁺CD38⁻ cells after 5 days in vitro expansion. CD34⁺CD38⁻ are putative stem cells, and our data indicate that cells are retained in their primitive state when miR-125a is overexpressed. We confirm the effect of reversal of miR-125a overexpression in human HSCs, as previously found in murine HSCs. In all cases the reversed cells behaved like the controls. While these results suggest that miR-125a is able to expand the number of CD34⁺ cells in vitro, while the number of most primitive CD34⁺CD38⁻ HSCs are maintained, we want to emphasize that they are preliminary as the experiment has not been replicated yet and thus must be interpreted with due caution.

Figure 4: Continuous expression of miR-125a is required to induce expansion of human HSCs. (A) Experimental setup. (B) Graphical representation of expression switch with Cre-LoxP system. (C) Percentage of respectively: living cells, marker⁺ (GFP or mCherry) cells, CD34⁺ progenitor cells and CD34⁺CD38⁻ stem cells, n=1. EV: Empty vector, EV-Cre: Empty vector + Cre excision performed, 125a: miR-125a overexpression, 125a-Cre: miR-125a reversed. (D) Total number of living cells based on cell counts of respectively: living cells, marker⁺ (GFP or mCherry) cells, CD34⁺ progenitor cells and CD34⁺CD38⁻ stem cells, n=1. EV: Empty vector, EV-Cre: Empty vector + Cre excision performed, 125a: miR-125a overexpression, 125a-Cre: miR-125a reversed.

DISCUSSION

In this chapter we explore the options for utilizing miR-125a as a tool for the expansion of human cord blood-derived HSCs. We show that miR-125a expression needs to be increased strongly to enhance HSC proliferation and maintain stem cell potential. We also show

that miR-125 expands cord blood-derived CD34⁺ cells in vivo ~120 fold and that these cells show a normal lineage contribution. We were however not able to show miR-125 had similar potential to induce expansion of iPSC-derived HSCs. Lastly, we confirm that cord blood-derived HSCs require continuous overexpression of miR-125a in order to govern the proliferative phenotype.

The in vitro expansion of HSCs is a topic of great interest in the experimental hematology field. On one hand it is key to increase HSC numbers, on the other hand it is beneficial to increase the engraftment potential of these expanded HSCs. Different approaches to achieve this goal can be divided into four main categories: cytokines and growth factors, small molecules that inhibit specific pathways or processes, synthetic culture media additives and lastly three-dimensional culturing techniques using scaffolds of different biomaterials and supporting cells.^{21,22} Cytokines and growth factors that support HSC maintenance, such as TPO, SCF and Flt3-L, are the most well defined category among these four previously mentioned. These approaches however have shown to only support HSCs in vitro for short periods of time.²³

In vitro expansion of human HSCs requires specific culture conditions that allow for the maintenance of stemness and prevent premature differentiation. Most in vitro cell culturing techniques rely on the addition of serum, a poorly defined component with a variable composition. Alternatives are the addition of commercially available serum replacing components. Continuous efforts are made to optimize culture conditions for primary cells, eliminating serum from these cultures in order to enhance reproducibility has been a major focus.^{24,25} A recent paper on the ex vivo expansion of murine HSCs uses a synthetic albumin replacement: Polyvinyl alcohol (PVA).²⁶ Another interesting finding in this methodological paper is the need for regular medium changes that are performed to wash out proinflammatory cytokines produced by the cells and avoid detrimental effects of these signaling molecules.

Results from this chapter show that miR-125a can be a potent tool for the expansion of cord blood derived HSCs. As with other expansion approaches, there are points of attention that will need to be addressed in order to successfully implement these new findings. In murine HSCs, the persistent overexpression of miR-125 often leads to the development of myeloproliferative disorders in serial transplantation studies.⁸ Their human counterparts however have not shown this detrimental phenotype in previous studies⁹ or the current study. An explanation for this phenomenon can be the differences in sensitivity to malignant transformation between murine and human cells. Murine cells need only a combination of two alterations that either activate oncogenes or inactivate tumor suppressing genes.²⁷ In human cells however need to collect many more additional oncogenic events to undergo malignant

transformation.^{28,29} For a potential clinical use of miR-125a as a tool for HSC expansion the tumorigenicity of these cells will need to be carefully investigated. To circumvent this potential drawback temporary overexpression of miR-125a to expand HSCs and subsequent reversal of miR-125a expression when sufficient expansion is reached may be optional. In figure 4 we show a pilot experiment where the reversal of miR-125a seems to revert the cells back to their initial state, comparable to the control conditions. Another strategy could be to further investigate HSC specific targets of miR-125a and pharmacologically inhibit these targets. This strategy is more flexible and potentially more suitable for clinical use.

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CONFLICT OF INTEREST DISCLOSURE

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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SUPPLEMENTAL

Table S1: Peripheral blood staining protocol for human cells.

Human PB stain **mOrange** (Canto-II compatible)

WBC			20x	10 µL/sample
CD3	APC-Cy7	2.5	50	
CD19	FITC	5	100	
CD33	BV421	0.625	12.5	
CD45	APC	2.5	50	
*CD34	PE-Cy7	4		
*CD38	BV605	4		

Live/death = PI

*optional colors require filter change on Canto-II

** addition of BV605 requires Brilliant stain buffer

RBC			20x	10 µL/sample
PBS	BSA 0.2%	9	180	
CD41a	PE-Cy7	0.1	2	
CD235a	APC	0.1	2	
CD42b	FITC	1	20

6 | Summary & Discussion

SUMMARY

The overall objective of the work in this thesis was to explore whether miR-125 could be employed to expand murine and human HSCs and to understand the mechanisms by which this would occur. We focused on investigating the reversibility of miR overexpression, identification of miR-125 targets and attempted to bridge a gap between murine and human model systems with the ultimate goal of being able to expand hematopoietic stem cells for clinical purposes.

In **Chapter 2** we discuss the role of miRs in the regulation of HSC self-renewal and differentiation throughout life and implications for future research. We conclude that multiple miRs have an indispensable role in HSC function. However, the exact roles of each of these miRs in processes specifically involved in maintaining HSC function during aging remain elusive. Gaining a better understanding of miR regulatory networks overall will enable us in the future to focus on specific and intricate processes such as hematopoietic aging.

In **Chapter 3** we investigate whether continuous overexpression of miR-125a is required to increase self-renewal, or rather whether temporary overexpression is sufficient. For this purpose, we developed a tool using the Cre-lox system. The Cre-lox system is a tool for controlling gene expression by recombination of genomic DNA. The Cre recombinase protein recognizes specific LoxP sites and based on their orientation can inverse, translocate, or delete the genomic sequence located in between two LoxP sites. We flanked the miR-125a sequence in our previously used retroviral overexpression plasmid with LoxP sites allowing for deletion of the genomic sequence in between, thus terminating overexpression. In order to easily distinguish overexpressing from reversed cells we added a fluorescent marker that marked overexpressing cells in green (with the fluorescent protein eGFP) and reversed cells in red (with the fluorescent protein mCherry). This resulted in a tool that can reliably overexpress miR-125a and of which the overexpression can be halted by expression of Cre recombinase at a chosen time point with a built-in fluorescent marker that allows for the analysis of the cells by flow cytometry. With the help of this tool, we show how continuous expression of miR-125a is necessary for the induction of HSC potential both in vitro and in vivo. Using RNA-sequencing we identify potential targets of the miR that are involved in molecular mechanisms that can be explanatory for the observed phenotype. In the current setup the efficient induction of Cre mediated excision of the LoxP flanked miR-125a sequence was a true technical challenge. In the experiment we relied on serial transplantations in which cells needed to be transduced and transplanted multiple times. Serial transplantations put significant strain on HSCs, every transplantation cycle subjects them to in vitro culture and the subsequent transplantation into the recipient pushes the generally quiescent cells to proliferate in order to repopulate the bone marrow.

Chapter 4 is aimed at better understanding the mechanism by which miR-125 exerts its effects on HSCs. In this chapter we focus on identifying mRNAs that are targeted by miR-125a in HSCs. We generated a new dataset with a method known as AGO2-RIP-seq. This approach combines immunoprecipitation (IP) of the AGO2 protein with subsequent RNA-sequencing. In our experiments we pulled down the protein complex that mediates the interaction between miRs and their target mRNAs, the RNA-induced silencing complex (RISC). We first performed IP in miR-125a overexpressing hematopoietic cells with an antibody directed against the main protein of the RISC complex, Argonaute-2 (AGO2), in such a way that the RNA in these samples remained intact. RNA was isolated from the IP samples and used as input for RNA-seq library preparation. This resulted in an RNA-seq dataset that was enriched for miR targets. In our attempt to integrate our experimental data with data from various different sources we highlight the variability between different experimental approaches for miR target identification. A major challenge in the use of large unbiased screening techniques for target identification is the inability to discern direct from indirect targets. This made it impossible to identify the cascade of different effects on mRNAs, proteins and feedback loops.

In **Chapter 5** we explore if our findings are also applicable to the human hematopoietic system. We establish an expression threshold that is needed to activate the miR-125 proliferative phenotype. In an in vivo xenograft experiment we found that overexpression of miR-125 can expand human CD34⁺ cells ~120 fold. We also attempted to enhance the function of iPSC-derived HSCs, which was unsuccessful so far. We performed a pilot experiment using human cord blood-derived CD34⁺ cells, with the same reversible expression plasmid that was used in murine cells in Chapter 3. We were able to reverse the miR-125a proliferative phenotype in human cells, indicating that the findings in murine HSCs in Chapter 3 may be applicable for human HSCs as well.

DISCUSSION

As highlighted numerous times in this thesis, our efforts converge to a main question that scientists in the hematological field ask: “How can we control self-renewal to expand hematopoietic stem cells (HSCs)?” I like to state the question exactly like this, to me the question is not ‘if’ but ‘how’, it will be only a matter of time before we discover the answers. In the light of this thesis, I would like to discuss three subjects that are important for the understanding of the current status of HSC manipulation for clinical purposes and indicate future directions for research. First, the different sources of HSCs that can be used, divided into two main categories: HSCs from existing sources and alternative sources such as embryonic or induced pluripotent stem cells. Secondly, the identification of targets for HSC expansion that have been successfully used to expand HSCs and my perspective on future efforts. And

lastly, the role of miR-125 as a tool for HSC expansion both by directly altering expression of miR-125 or by mimicking the miRs beneficial effects on downstream pathways.

Various sources of HSCs

In order to expand HSCs for clinical purposes, we must first consider the possible sources of starting material. These can be divided into two categories: existing sources of HSCs or the generation of HSCs from alternative sources.

Existing sources of HSCs comprise the bone marrow, cells isolated from the peripheral blood after mobilization from the bone marrow, or cord blood-derived CD34⁺ cells. In 1957 the first successful HSC transplantation was performed by intravenous infusion of donor bone marrow from a monozygotic twin to his sibling.¹ Roughly 30 years later the first successful transplant with cord blood derived HSCs was successfully performed, in which the cord blood-derived HSCs from the patient's sibling were used.² More than 30,000 patients in the European Union received an HSCs transplant yearly in 2017 and 2018.^{3,4} One of the advantages of cord blood-derived HSCs is that they are easily obtained from the blood that remains in the umbilical cord and placenta after delivery that is otherwise discarded. For existing sources of HSCs, it is important to carefully match donor and recipient based on human leukocyte antigen (HLA) haplotypes. When HLA haplotypes do not match, the recipient will be at risk of developing graft versus host disease (GVHD) or graft failure.⁵ Again, cord blood-derived HSCs have an advantage, as these cells are not yet immunologically mature and therefore can be matched to a recipient more easily.^{6,7} A major drawback however is the total number of cells that can be obtained from a single donor, this is often not sufficient to allow for successful transplantation of an adult. Selecting two adequately HLA-matched cord blood units might aid in overcoming this problem.⁷

Alternative sources of HSCs could be embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). In 1981, Evans et al. were able to culture pluripotent stem cells isolated from mouse embryos.⁸ It took almost 20 more years for the first ESC-derived cell line to be isolated from human blastocysts.⁹ The use of ESCs from human embryos raises significant ethical concerns. A solution to these concerns became within reach when a technique was discovered that reprograms somatic cells into iPSCs.^{10,11} In the case of HSC transplantation, this technique also offers a solution to the difficulty of finding an HLA matched donor. Fibroblasts from a patient can be reprogrammed into iPSCs and differentiated into autologous transplantable HSCs. Autologous transplantation has the advantage of a full HLA match; however, all potential genetic vulnerabilities are also still present. Thus, mutations that might predispose the HSCs to develop hematological disease are also still present in the generated stem cells.

Tools necessary for HSC expansion

In vitro generation or expansion of HSCs does not only need a source from which HSCs can be reliably obtained. Technically there are still some challenges that need to be overcome.

Firstly, we are in need of culturing methods that allow for the proper support of HSC expansion, regardless of their source. A considerable difficulty in the field has been to maintain HSCs *ex vivo*, when removed from the bone marrow niche HSCs will quickly differentiate and lose their stem cell potential. New culturing techniques combining specific media supplements and simulating a 2D microenvironment make it possible to retain murine HSCs in vitro for 16-20 weeks.¹² It has also shown to be possible to generate erythroid, megakaryocytic and myeloid cells from iPSC cell lines in vitro.¹³ The generation of true HSCs with a long term engraftment potential remains difficult. For the generation of HSCs from ESCs it has been shown that the spatial arrangement plays a role in the development of hematopoietic cells.¹⁴ Knowledge obtained from studies on ESC development can be harnessed to optimize in vitro differentiation methods for iPSCs and the knowledge from these studies can be applied to iPSCs.^{15,16} A recent study shows the development of HSCs by differentiating iPSC-derived hemogenic endothelial in vivo in fetal sheep liver.¹⁷ This discovery potentially paves the way for in vitro generation of iPSC derived HSCs.

The second prerequisite is a toolkit that will allow for the repair of genetic errors such as mutations that predispose to the development of malignancies, or mutations in key genes that result in non-malignant hematopoietic diseases such as sickle cell anemia. With the discovery of the CRISPR/Cas9 gene editing system we are now able to precisely edit the genomic material in many different cells, among which human HSCs.¹⁸⁻²⁰ In the future we should be able to obtain somatic cells from a patient, reprogram these cells into iPSCs, repair essential genes and differentiate these cells into transplantable HSCs.

Targets for the expansion of HSCs

HSCs can be maintained in vitro using standard protocols that use a cocktail of cytokines that only allow for short term culture. As mentioned above, methods have been developed to extend the *ex vivo* HSC culturing period to up to 20 weeks, whilst still losing a portion of the cells along the way.¹² The main hurdle in culturing HSCs is preventing them from differentiating and thus losing their true stem cell identity. If the goal is to expand HSCs for clinical purposes, we must also keep in mind the high standards that apply to the manufacturing of products for clinical use. In the case of a culture protocol, all components must be standardized and results must be reproducible. It would therefore be most desirable to add defined components such as a small molecule that enhances a self-renewal program in the cultured HSCs. Different culture media supplements have been successfully used to expand HSCs in vitro.²¹ Examples are StemRegenin-1 (SR-1), an aryl hydrocarbon receptor

antagonist that has been shown to successfully expand cord blood derived CD34⁺ cells in a phase I/II clinical trial.^{22,23} Another small molecule that has shown promising results in expanding cord blood-derived HSCs is UM171²⁴, which has been successfully used in a patient and in a phase I/II clinical trial.^{25,26} Other compounds are aimed at epigenetically modifying HSCs such as the histone deacetylase inhibitor valproic acid²⁷ and UNC0638, a small molecular inhibitor of the DNA methyltransferases G9a/GLP.²⁸

Ideally, we should be able to develop a small molecule or cocktail of small molecules that can be added to a standard culture media as a 'HSC expansion supplement'. Or maybe even develop two different flavors, an HSC expansion and an HSC maintenance supplement, where the latter can be added to maintain HSCs in their naturally dormant state. (Figure 1) When the need arrives, suitable donor material can be selected and cells can be expanded in vitro in order to increase the chances of a successful engraftment. In the case where no suitable donor material is available, we can switch to plan B in which fibroblasts from the patient are reprogrammed to iPSCs and differentiated into HSCs. A likely intermediate

Figure 1: Approaches to HSC transplantation with the availability of HSC expanding agents that can be added as a cell culture supplement. Left: 'Plan A', Approach for allogeneic transplant with donor HSCs. Right: 'Plan B', Approach for autologous transplantation of iPSC-derived HSCs including a gene editing repair step for the newly generated HSCs.

step is to genetically engineer these new HSCs in such a way that they will not succumb to the same disease as the 'old' HSCs. In which case an HSC maintenance media supplement would be of great help. The refitted HSCs can then be expanded and transplanted back into the patient. With the added benefit that these cells are exactly HLA matched.

MiR-125 as a tool to expand HSCs

MicroRNA-125 (miR-125) has shown to be a very potent enhancer of HSC function. This miR has sparked interest when it was first discovered.^{29,30} Further studies showed that overexpression of miR-125 gave HSCs a proliferative advantage over their wild type counterparts.^{31–33} In this thesis we showed how we can also efficiently reverse the effects of miR-125 by reversing the overexpression.³⁴ This last finding is important for the further development of miR-125 as a tool for HSC expansion. We have discovered that the effects induced by miR-125 are reversible, and not the result of a reprogrammed-like state. This opens the possibility to find a way to mimic the effect of miR-125 overexpression in a short-term culture, allowing for the expansion of HSCs without permanent genetic alterations. A more sophisticated method of activating and inactivating miR-125 overexpression might aid to a more elaborate characterization of the effects of miR-125a overexpression reversal on HSC function.

In Chapter 5 we have shown how miR-125a overexpression also enhances HSC function cord blood derived CD34⁺ cells both in vitro and in vivo. Additionally, we have attempted to utilize miR-125a to enhance the function of iPSC-derived CD34⁺ cells. Unfortunately, we were not able to demonstrate engraftment from iPSC-derived cells. There are various explanations possible of why we were not able to show engraftment of iPSC-derived miR-125a overexpressing CD34⁺ cells. First, multiple factors of this experiment are eligible for optimization. Lentiviral transduction of these cells might not have been well tolerated, although they were very effective as shown by the reported transduction efficiencies. We have not been able to investigate whether the miR-125a overexpression needs to be timed to a specific stage of differentiation of the iPSCs into CD34⁺ HSPCs. It is important to reiterate that successful engraftment of iPSC-derived HSCs has not been previously achieved. Finally, we also show how the effect of miR-125a overexpression, like in murine HSCs, is reversible. This would mean that temporary inhibition of miR-125a targets is sufficient to enhance the function of human HSCs. Creating opportunities for clinical use of the miR-125 mechanism.

The expression of miRs is deregulated in many diseases. It is no surprise that efforts have been made to utilize knowledge on miRs and turn them in to therapeutics. MiR-based therapeutics aim at exploiting miRs with tumor suppressing functions, or inhibiting specific miRs by antimiRs.³⁵ Difficulties remain in ensuring the stability and effective delivery of

these types of therapeutics. In the case of utilizing miR-125 to expand HSCs, it may be best to focus on the downstream molecular pathways that are responsible for the observed phenotype in HSCs. For the purpose of HSC transplantation, cells of interest can be subjected to in vitro expansion without the need for in vivo delivery of miR based therapeutics. In this thesis we have made efforts towards identifying the miR-125a targets responsible for the expansion of HSCs. Potentially interesting targets that were previously identified are PTPN1 and MAPK14 (also known as p38).³² PTPN1 is part of a family of signaling molecules that plays a role in various cell processes, of which cell cycle and differentiation are of particular interest in HSC biology. Due to PTPN1's role in insulin regulation it is also an interesting target for the treatment of diabetes mellitus type 2 and efforts have been made towards developing an inhibitor.³⁶ MAPK14 is a pathway that plays a role in a plethora of diseases such as cancer, inflammatory disease and cardiac disorders. For this protein many different inhibitors have been developed.³⁷ Most of these compounds have failed to pass clinical testing due to off target effects, they could however still be interesting candidates for in vitro use where these off-target effects pose less of a concern. In chapter 3 we identify a number of novel miR-125a targets which require further investigation. For the protein GLS which was found to be most significantly downregulated upon miR-125a overexpression, a promising inhibitor compound has already reached phase I clinical trials.³⁸ As stated before it might be possible to develop a cocktail of different inhibitors of key miR-125 targets that together can mimic the effect of miR-125 overexpression without the need for genetic perturbations. Further characterization of the novel targets that we have identified will provide more insight in the molecular mechanisms involved. Ideally, we can then develop a strategy that specifically blocks or activates the pathways involved. Inhibitory compounds can then be added to an in vitro culture and in that way facilitates HSC expansion. Future efforts should aim towards unraveling the molecular mechanisms by which miR-125 governs its beneficial effects on HSCs.

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A

List of abbreviations

Nederlandse samenvatting

Acknowledgements

Curriculum Vitae

LIST OF ABBREVIATIONS

<i>Acronym</i>	<i>Clarification</i>
AGO2	Argonaute-2
CAFC	Cobblestone area forming cell
CFU	Colony forming unit
Epo	Erythropoietin
ESC	Embryonic stem cell
EV	Empty vector
FACS	Fluorescence-activated cell sorting
G-CSF	Granulocyte colony-stimulating factor
GFP	Green fluorescent protein
GVHD	Graft versus host disease
HLA	Human leucocyte antigen
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
IP	Immuno precipitation
iPSC	Induced pluripotent stem cell
ITSX	Insulin-Transferrin-Selenium-Ethanolamine
LTC-IC	Long term culture initiating cell
miR	MicroRNA
MPP	Multipotent progenitor
mRNA	Messenger RNA
MS	Mass-spectrometry
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cell
PVA	Polyvinyl alcohol
RBC	Red blood cell
RISC	RNA induced silencing complex
RNAi	RNA interference
SCF	Stem cell factor
SILAC	incorporation of stable-isotope labeled amino acids into cellular proteomes
TPO	Thrombopoietin
UTR	3' untranslated region
WBC	White blood cell
WT	Wild type

NEDERLANDSE SAMENVATTING

Het bloed dat door de bloedvaten van het menselijk lichaam stroomt kan worden beschreven als zowel een vloeistof als een weefsel en is op die manier zeer opmerkelijk. Het bloed bestaat uit verschillende soorten cellen. Verschillende bloedcellen hebben ieder hun eigen specifieke functie. Rode bloedcellen transporteren gassen zoals zuurstof en koolstofdioxide, witte bloedcellen zijn een deel van het immuunsysteem en de bloedplaatjes zorgen ervoor dat er stolling optreedt bij de beschadiging van een bloedvat. Deze zogeheten volwassen bloedcellen hebben niet het eeuwige leven. Rode bloedcellen leven ongeveer 120 dagen en bij witte bloedcellen kan dit variëren tussen enkele uren en meerdere dagen. Het is dus belangrijk dat deze cellen continue aangevuld worden om de functie van het bloed te behouden. Hematopoëtische stamcellen (HSC's) zijn gespecialiseerde stamcellen die verantwoordelijk zijn voor het aanvullen van de verschillende bloedcellen gedurende een mensenleven.

Het proces waarbij stamcellen nieuwe bloedcellen aanmaken heet hematopoëse. De hoofdrolspeler in dit proces is de HSC. Deze stamcellen zitten in het beenmerg en kunnen zowel zichzelf vernieuwen als zichzelf verder ontwikkelen naar verschillende zogenaamde voorloper-cellen. Deze voorloper-cellen kunnen zich op hun beurt weer verder ontwikkelen naar bijvoorbeeld een witte bloedcel. Dit proces wordt differentiatie genoemd. Deze differentiatie kan alleen in één richting plaatsvinden, een voorloper-cel kan dus niet terug veranderen in een stamcel. Het proces dat plaats vindt in de stamcellen om te besluiten of zij zich wel of niet moeten gaan differentiëren is strak geregeld. Als cellen te veel differentiëren blijven er niet genoeg stamcellen over, als ze te weinig differentiëren worden de volwassen bloedcellen niet op tijd aangevuld.

Volwassen HSC's worden op twee manieren beïnvloed om het keuzeprocess tussen wel of niet differentiëren te regelen. De eerste manier zijn de extrinsieke factoren, dit zijn invloeden vanuit de omgeving van de cel. Dit kunnen bijvoorbeeld signalen zijn die afkomstig zijn van andere cellen in het beenmerg die de HSC's omringen. De tweede manier zijn intrinsieke factoren, dit zijn processen die binnen in de cel zelf plaatsvinden. De uiting van verschillende genen heeft invloed op de eiwitten die zich in de cel bevinden en kan zo het gedrag van de cel beïnvloeden.

Net als alle andere weefsels in ons lichaam is ook het hematopoëtische systeem onderhevig aan veroudering. Oude stamcellen functioneren minder goed dan jonge stamcellen. Oude stamcellen kunnen minder goed nieuwe bloedcellen aanmaken en raken gemakkelijker ontregeld waardoor problemen kunnen ontstaan zoals bloedkanker (leukemie) of een tekort aan bepaalde bloedcellen. Deze problemen worden vooral toegeschreven aan de intrinsieke factoren die de functie van de HSC's beïnvloeden.

Een van de intrinsieke factoren die de functie van HSC's beïnvloeden zijn microRNA's (miR's). Een miR is een heel klein stukje genetische code (RNA) dat niet vertaald wordt naar een eiwit, een niet-coderend RNA. Normaal gesproken wordt een gen actief gemaakt, waarna een stuk van de genetische code (messenger RNA) gekopieerd wordt van het DNA. Dit stuk messenger RNA wordt door de cel verwerkt en omgezet in een eiwit. Een eiwit kan allerlei functies vervullen in de cel en er bijvoorbeeld voor zorgen dat een HSC zichzelf gaat delen. Een miR kan grotere RNA's herkennen en deze vervolgens blokkeren. De miR zorgt er dan voor dat het RNA dat hij blokkeert geen eiwit kan worden. Op deze manier kan een miR invloed hebben op de aanwezigheid van verschillende eiwitten en op die manier ook het gedrag van een cel.

Er zijn veel verschillende miRs, welke allemaal hun eigen nummer gekregen hebben. MiR-125a is bijzonder interessant omdat mijn voorgangers al hebben ontdekt dat deze miR ervoor kan zorgen dat HSC's beter gaan presteren. Dit is onderzocht door een genetische modificatie aan te brengen in HSC waardoor miR-125a verhoogd tot expressie komt, dit wordt overexpressie genoemd. HSC's met overexpressie van miR-125a groeien sneller en maken meer volwassen bloedcellen aan dan HSC's zonder genetische modificatie. Mogelijkerwijs kan het mechanisme dat achter deze miR schuilt gebruikt worden om van een beperkte hoeveelheid stamcellen een grotere hoeveelheid stamcellen te maken. Dit kan van grote waarde zijn voor het verbeteren van stamceltransplantaties. Donor-materiaal is veelal schaars en de kans op een succesvolle transplantatie wordt hoger naarmate het aantal stamcellen dat getransplanteerd wordt hoger is.

In deze thesis onderzoek ik samen met collega's hoe miR-125a precies werkt in HSC's en welke mechanismen er achter de zichtbare effecten op het gedrag van de HSC's schuilen. We onderzoeken of de effecten die overexpressie van miR-125a op HSC's heeft omkeerbaar zijn, proberen de doelwitten (ook wel targets) van miR-125a te identificeren en proberen de kloof tussen het muismodel en de mens te overbruggen. Het ultieme doel hierbij is om van weinig HSC's veel HSC's te kunnen maken voor klinische doeleinden.

In **hoofdstuk 2** bediscussiëren we de rol van miRs in het reguleren van de keuze van een HSC om zichzelf te vernieuwen of te differentiëren wanneer de cellen ouder worden. En gaan in op de gevolgen die dit kan hebben op toekomstig onderzoek. We concluderen dat meerdere miR's een onmisbare rol hebben in het functioneren van HSC's. Helaas blijven de exacte rollen van de individuele miR's op het onderhouden van de HSC's moeilijk vast te stellen. Als we eerst een beter beeld kunnen krijgen van de algemene netwerken waarin miR's hun invloed uitoefenen, kunnen we in de toekomst specifiek op processen zoals veroudering van het hematopoëtische systeem focussen.

In **hoofdstuk 3** onderzoeken we of continue overexpressie van miR-125a nodig is om HSC's meer te laten zelf-vernieuwen. Of dat een tijdelijke aanwezigheid van miR-125a hetzelfde effect geeft. Hiervoor hebben we een genetisch gereedschap ontwikkeld dat gebaseerd is op een eiwit dat specifieke stukjes in het DNA kan herkennen, Cre-recombinase. Dit eiwit wordt veelvuldig gebruikt om genexpressie te controleren. Het Cre-recombinase eiwit herkent specifieke stukjes in het DNA die Lox-P sites genoemd worden. Afhankelijk van hoe deze Lox-P sites georiënteerd zitten op het DNA kun je het stukje DNA dat ertussen zit omdraaien, verplaatsen of weghalen. In ons geval hebben we twee Lox-P sites aangebracht rondom het stuk DNA dat voor miR-125a codeert. Dit stelde ons in staat om miR-125a weg te kunnen knippen op een door ons gekozen moment. Hier hebben wij ook een code voor twee verschillende fluorescerende eiwitten aan toegevoegd. Een groene: GFP en een rode: mCherry. Het groene eiwit wordt aangemaakt als miR-125a 'aan' staat, en als we het weg knippen wordt het rode eiwit aangemaakt om aan te geven dat miR-125a 'uit' staat. Met de hulp van dit stukje gereedschap laten we zien dat het effect dat miR-125a heeft op HSC's omkeerbaar is. We doen dit zowel in cellen die in het lab gekweekt worden als in muizen die een beenmergtransplantatie hebben ondergaan. Daarnaast is er ook een analyse uitgevoerd op het RNA in de cellen om te ontdekken welke genen verantwoordelijk zijn voor het gedrag van de HSC's dat we observeren.

In **hoofdstuk 4** duiken we verder in het mechanisme waarmee miR-125a zijn effecten uitoefent op HSC's. We proberen verder te specificeren welke messenger RNA's (mRNA's) de doelwitten (of targets) zijn van miR-125a. Hiervoor hebben we een dataset gegenereerd met een methode die bekend staat als: AGO2-RIP-seq. Met deze methode konden we specifiek de mRNA's uit de cellen isoleren die het doelwit zouden moeten zijn van miR-125a. Vervolgens hebben we de verkregen data vergeleken met datasets uit andere bronnen. Hiermee laten we zien dat er veel variatie is tussen verschillende methodes om mRNA doelwitten van miR's vast te stellen. Een complicerende factor bij het zoeken naar doelwitten van miR's is het onvermogen om onderscheid te maken tussen directe en indirecte doelwitten van de miR. Aanvullend hebben we onderzoek gedaan naar de manier waarop de miR zijn doelwitten herkent. Hiervoor hebben we kleine aanpassingen (mutaties) gemaakt in de genetische code van de miR. We hebben voor verschillende mutaties getest of het effect van de miR op het gedrag van de cellen hetzelfde bleef. Voor een bepaald stukje van de genetische code hebben we aangetoond dat deze onmisbaar is. Als in dit gedeelte een mutatie wordt aangebracht kan de miR er niet meer voor zorgen dat de cellen versneld gaan delen. Dit is een belangrijk puzzelstukje in het begrijpen van het mechanisme waarmee miR-125a zijn effecten uitoefent op HSC's.

In **hoofdstuk 5** ontdekken we of de behaalde resultaten in muizencellen ook van toepassing zijn op humane cellen. We gebruikten hiervoor humane HSC's die afkomstig zijn uit

navelstrengbloed. Het bloed dat na de geboorte overblijft in de placenta en de navelstreng verzamelden wij en hieruit isoleerden wij de HSC's op basis van een receptor op het celoppervlak: CD34. Deze cellen noemen wij dan ook CD34 positieve cellen. We stellen vast dat er een drempel is waar de expressiegraad van miR-125a boven moet komen voor de effecten ook in humane CD34 positieve cellen zichtbaar worden. Ook stellen wij vast dat door overexpressie van miR-125a er ongeveer 120 keer meer humane CD34 positieve cellen te zien zijn wanneer deze cellen getransplanteerd worden in muizen. Dit is ten opzichte van normale HSC's die geen overexpressie van miR-125a hebben. We proberen ditzelfde ook te doen met HSC's die afstammen van geïnduceerde pluripotente stamcellen maar helaas zonder succes. Deze speciale stamcellen worden in het lab gemaakt door een ander soort cellen (bijvoorbeeld van fibroblasten uit de huid) met een specifieke techniek te herprogrammeren. Als laatste is onderzocht of in humane cellen, net als in de muizen cellen, het effect van miR-125a omkeerbaar is en de eerste bevindingen wijzen erop dat dit het geval is.

Tot slot bediscussiëren wij hoe wij deze kennis in ons voordeel kunnen gebruiken om controle te krijgen over de processen die bepalen of HSC's gaan zelf-vernieuwen of differentiëren. Met als ultieme doel om HSC's te expanderen voor klinische toepassingen. We bespreken de verschillende bronnen van HSC's die gebruikt zouden kunnen worden, zoals geïnduceerde pluripotente stamcellen (iPSC's). Welke gereedschappen en technieken nodig zijn. En welke genen belangrijke doelwitten zijn, waarbij uiteraard miR-125a een goede kanshebber is. Naar mijn mening is dit niet de vraag of het gaat lukken, maar wanneer het gaat lukken. Het lijkt mij zeer waarschijnlijk dat het slechts een kwestie van tijd is voor wij deze technieken kunnen ontwikkelen.

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breaks. **Laura**, I wish you good luck on wrapping up your thesis and hope you will be able to defend in the near future! You got this! **Klaske**, when I moved into my new house, I had some trouble settling in. To make matters worse, I had not yet found a sofa that could fit up the staircase. You made it your personal mission to get me that sofa. We headed off to IKEA and managed to squish everything - including ourselves - into my tiny car. With some funny pictures as a result. Thank you so much for helping me out, and in general just being a fun colleague to be around. **Amanda**, I hope Arthur stopped reading by now because I have to be honest, you are my favorite Brazilian person. It was fun sharing your room for a few days when I was getting ready to move into my new house. Also, your hugs always made my day 100x better. Now that you are back in Brasil, I hope we get to meet each other again one day. **Christy and Andrea**, thank you for all the fun times. **Christy**, I really hope we can still make a great wedding party for you in the near future! Thank you for hosting my compulsory birthday party that got completely out of hand on a weekday. Even the shared hang over was sort of fun... **Andrea**, it was cool to see someone from the same university joining the ERIBA squad. Thank you for your lovely company and of course for looking after my cats multiple times when I was out of town. **Michael**, my life safer when it came to some of my sequencing adventures but also many other PhD struggles. Thank you for all the lovely dinners, and yes you cooked most of them. But I hopefully repaid some of that with helping you move to Amsterdam where I hope you will find some great opportunities for your scientific career.

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Two other facilities of the UMCG that I was a regular visitor of are the **CDP** (Centrale Dienst Proefdieren) and the **Flow Cytometry Unit**. Thank you to all the CDP staff for taking great care of my animals. And a very big thank you to **Geert, Johan** and **Theo** from the cytometry unit, I did some math and figured out that I have registered over 400 hours of sorting time of which most took place in the final two years of my PhD. That is a lot of hours spent at the cell sorters. Luckily, we never ran out of conversation material. Thank you for helping me get those last cells out of my samples.

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CURRICULUM VITAE

Education

- 2016 - 2020 PhD Candidate, European Research Institute for the Biology of Ageing (ER-IBA) Groningen, NL
 Thesis: MicroRNA-125a in Hematopoiesis
 Research group: Ageing Biology & Stem Cells; Principal investigator: Prof. Dr. Gerald de Haan
- 2012 - 2015 MSc Cardiovascular Research, VU University Amsterdam, NL
 Optional courses: Advanced cardiac diagnostics, Academic Teaching & Presenting and Bio business
- 2009 - 2012 BSc Biomedical Sciences, VU University Amsterdam, NL
 Minor: Evolutionary Biology and Ecology
- 2002 - 2008 Pre-university secondary education, BSG Bergen, NL
 Subjects: Biology, Chemistry, Physics, Math, Dutch, English, German and French

PhD Project

Short overview of lab techniques:

Basic molecular work; Cell culture; Virus work (ML-II); Flow Cytometry; Laboratory animals (mouse); Human primary cells; Immunoprecipitation; RIP-seq and RNA-seq.

Courses and Certificates:

- Laboratory Animal Course (Article 9)
- Research Ethics
- Managing your PhD (Project Management)
- Good Clinical/Laboratory Practice (GCP/GLP)
- Statistics for Biologists

Awards and activities:

- Best Elevator Pitch, CRCG 2018
- PhD Activity Committee, ERIBA 2016-2020
- Student Council, CRCG 2017-2019

Conferences:

2016 - 2019: Cancer Research Center Groningen (CRCG) PhD Student meeting
 2018 and 2019: International Society for Experimental Hematology
 2016 and 2017: Dutch Society for Stem Cell Research (DSSCR)
 2017 and 2019: Molecular Biology of Aging meeting

Previous Research Projects

02/2015 - 04/2015	Literature review: Circulating biochemical markers linking post-acute myocardial infarction ventricular remodeling to diabetes mellitus type II and obesity.
Supervisor:	Dr. Tineke van der Pouw Kraan (VUmc)
03/2014 - 11/2014	Major internship MSc: Identification of Cardiac Conduction System Specific Enhancers. Department of Anatomy, Embryology and Physiology, AMC Amsterdam
Supervisors:	Prof. Dr. Vincent Christoffels (AMC) Prof. Dr. Reina Mebius (VUmc)
04/2014 - 07-2014	Minor internship MSc: CD27/CD70 interactions in atherosclerosis. Department of Medical Biochemistry, AMC Amsterdam
Supervisors:	Prof. Dr. E. Lutgens (AMC) Prof. Dr. A.J.G. Horrevoets (VUmc)
09/2012 - 12/2012	Internship BSc: Selective Starvation of Cancer Cells; Quantifying the Warburg Effect Department of Molecular Cell Physiology, VU University Amsterdam
Supervisor:	Prof. Dr J. Lankelma (VU)