The functional characterization of microRNA-125 in hematopoiesis

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SUMMARY AND DISCUSSION

MiRNAs effectively fine-tune gene expression and thereby regulate many cellular processes such as apoptosis, proliferation and differentiation. Their potential to target hundreds of transcripts at the same time provides an opportunity to study this collective effect by modulating a single miRNA. In this thesis we aimed to functionally characterize the role of miR-125 family in blood cell production and gain mechanistic insight in targets that are recognized by this important microRNA.

Chapter 2 serves as an introduction to the field of hematopoietic stem cell biology, and provides basic definitions, a description of the hematopoietic hierarchy, methods for prospective isolation of HSC, and widely used in vivo assays. In the second part of this Chapter we describe changes that occur in the hematopoietic system upon ageing at the cellular and molecular level. Data concerning the role of miRNAs in hematopoietic stem cell ageing are still limited, however the phenotypes induced by some miRNAs can partially phenocopy ageing-related changes. This suggests that there may be a role for miRNAs in at least some aspects of the aging process, which will emerge in the near future.

In Chapter 3 we characterize miRNAs involved in the regulation of hematopoiesis at different levels of differentiation. We highlight the interplay between miRNAs and key transcription factors that regulate blood cell formation. Moreover, we describe how miRNAs are regulated and how perturbations of their expression can lead to development of hematological malignancies. The miR-125 family is of particular importance as it is highly expressed in HSCs and in several types of leukemias.

In Chapter 4 we have systematically compared each of the three members of the miR-125 family: -a, -b1 and -b2. All three miRNAs share the same seed sequence, but differ in their 3’UTR (differences between –a and –b1/b2) and their chromosomal location, implying different regulation of the transcription of each miRNA family member. Analyses of CFU-GM, CAFC and liquid cultures assays indicated very similar effects of overexpression of each miR-125 family member on hematopoiesis. Ectopic expression of any member led to a proliferative advantage, increased frequency of cobblestone area forming cells and strongly enhanced replating activity, suggesting expansion
of myeloid progenitors with clonogenic potential. Transplantation experiments also revealed very similar effects of each miR-125 family member, such as splenomegaly and increased bone marrow cellularity, myeloid skewing represented by increased numbers of myeloid cells accompanied by decreased red blood cell counts in blood. Interestingly, we observed a gradual decrease in the frequency of phenotypic LT-HSC in the course of transplantation experiments. Six months post transplantation almost no phenotypical stem cells could be observed in the bone marrow of transplanted mice, while a novel population of Lin-Sca-1-c-Kit cells emerged. Nevertheless, serial transplantation of whole bone marrow cells resulted in the repopulation of lethally irradiated secondary recipients, documenting the presence of functional stem cells. In some cases we observed development of myeloproliferative neoplasms in reconstituted recipients. Therefore, we conclude that all three miR-125 family members interfere with the expression of c-Kit and/or Sca-1 (although none of them is a predicted direct target of miR-125) leading to the loss of phenotypical but not functional LT-HSCs. Overall, features of the hematopoietic phenotype were similar in mice transplanted with HSPCs overexpressing miR-125a, -b1 or -b2, and only mild quantitative differences were observed between these three miRNAs. To validate the importance of the seed sequence we performed experiments with a seed sequence mutant of miRNA-125a, where only one nucleotide has been changed in the 5p arm. This mutation did not change the secondary structure of the precursor-miRNA and had no effect on its processing. We did not observe any increased repopulating potential or proliferative advantage in cells overexpressing this seed sequence mutant compared to the control cells. To further support our observations on the crucial role of the miR-125 seed region, we also performed experiments where miR-125 was repressed in LT-HSCs using an antagonir. In these experiments we observed a deleterious effect of miR-125 downregulation manifested by the loss of cells with decreased miR-125 levels in blood at early time points and their absence in bone marrow 24 weeks post transplantation.

Overall, our experiments show that all three miRNA-125’s similarly induce self-renewal activity in a seed sequence dependent manner. Long-term follow up of mice repopulated with miRNA-125 overexpressing HSPCs showed that a fraction of these recipients developed myeloproliferative syndromes. During the last decade many groups aimed to decipher the rules for miRNA target recognition (Bartel, 2004; Fabian et al., 2010) which led to the conclusion that the seed region is playing a pivotal role in this process. The current bioinformatics predictions of putative miRNA targets mainly rely on perfect seed region pairing, and to a lesser extent on miRNA 3’ end pairing with the target. These predictions generate large networks of interactions, but it should be realized that experimentally only a small group of these target networks have been functionally validated. Keeping in mind the differential expression of miRNAs and their putative targets at the different stages of hematopoiesis, it remains very challenging to evaluate cause-versus-consequence effects, and to assess the relative degree of potency in exerting biological effects. This may have contributed to the supposedly contradictory reports concerning the miR-125 family (Gerrits et al., 2012; Guo and Scadden, 2010; O’Connell et al., 2010; Ooi et al., 2010). In addition, these seemingly contradictory roles may have originated from the use of different cell populations, different family members and the genetic context (overexpression of miR-125 alone, or rather the whole cluster of miR-99b, let-7e and miR-125a) (Gerrits et al., 2012; Guo and Scadden, 2010; O’Connell et al., 2010; Ooi et al., 2010). The results presented in Chapter 4, which documents a very high degree of similarity between all family members proofs the importance of the seed sequence for the miR-125 induced phenotype in HSPCs. The inclusion of a seed sequence mutant of miR-125a has turned out to be a powerful tool in our attempts to separate several independent effects induced by the same miRNA.

In Chapter 5 we questioned whether miRNA-125a would be able to induce self-renewal and long-term repopulating ability in hematopoietic progenitor cells, that normally do not display such potential. We found that indeed, not only LT-HSCs but also a population of Lin-Sca-1-c-Kit+ cells, stringently depleted from LT-HSC, contains long-term repopulating potential in serial transplantation experiments upon enforced overexpression of miRNA-125a. This was accompanied by an increased lifespan of these cells. Interestingly, these ‘long-lived progenitors’ provided multilineage repopulation in myeloid and lymphoid lineages. By applying a cellular barcoding method we determined that ~6 to ~20 clones contributed to blood cell production (each clone contributing more than 0.5% to the Gr-1+ cell population) per mouse transplanted.
with miR-125 overexpressing progenitors, indicating a polyclonal repopulation. It is formally possible that the population of FACS-purified progenitors used for transductions potentially contains ‘contaminating’ LT-HSCs that could gain a proliferative advantage upon miR-125a OE. This scenario is possible, however not supported by the presented data on polyclonal repopulation of mice transplanted with miR-125a OE progenitors or the limiting dilution results (indicating similar frequency of repopulating cells in control LT-HSC and progenitors with miR-125a OE). Interestingly, a similar effect of miR-125 overexpression was observed in human LT-HSC and MPPs isolated from CB. These cells showed increased self-renewal and life-span. In contrast to our observations in murine cells, in human cells overexpressing miR125 no cases of hematological malignancies were observed in xenotransplanted recipients. This implies that an evolutionary conserved pathway, controlled by miR-125, regulates self-renewal in mouse and human cells and suggests that miR-125 targeted transcripts need to be repressed in HSPCs to maintain their potential.

To gain more insight in the molecular mechanism of miR-125 mode of action we performed stable isotope labelling in cell culture (SILAC) using a myeloid cell line (32D cells). In our search for targets we did include a seed sequence mutant to control for off-target effects and to exclude all targets similarly affected by miR-125a and the mutant. We identified a limited set of targets and pathways repressed upon miR-125a OE that are likely differentially expressed in LT-HSC and progenitors. Probably this differential expression functionally distinguishes these two cell populations. Therefore, our finding offers insight into the key molecular differences which render stem cells from progenitors.

**FUTURE PERSPECTIVES**

In this thesis we aimed to characterize the role of the three members of the miR-125 family in hematopoiesis and to assess the potential of miR-125 to expand LT-HSC and progenitors as a possible approach to generate or expand HSC *in vitro*. In the next paragraphs we will discuss remaining questions and suggest future areas of research.

**THE EFFECTS OF OVEREXPRESSION OF MIR-125 FAMILY MEMBERS AND THE IMPORTANCE OF THE SEED SEQUENCE**

We find that the hematopoietic phenotype induced by overexpression of each of the miR125 family members is highly similar. This underscores the importance of the seed sequence, which is shared by all three family members. However, it is likely that endogenous expression of each member of the family is regulated differently as each gene is embedded in a distinct genetic context. Thus, it is possible that quantitative mass spectrometry (iTRAQ) (Wiese et al., 2007), combined with recently developed techniques such as CLASH-CLIP and improved CLEAR-CLIP (Helwak et al., 2013; Moore et al., 2015) will allow to determine *in vivo* transcripts that are bound by miRISC complexes in various blood cell populations. These types of experiments may validate the normal physiologic relevance of the targets that we identified in the experimental setup with 32D cell line and unravel new, important targets.

Although mutant miR-125a was not able to induce self-renewal in LT-HSCs or in progenitors, some phenotypes were in fact induced upon its overexpression. For example, in mice transplanted with the seed sequence mutant we observed a decreased frequency of megakaryocytic and erythroid progenitors (MEP), which was in between the frequencies of MEPs in mice transplanted with control or miR-125 overexpressing LT-HSC. Thus, the mutant can be used as a filtering criterion to separate seed-sequence dependent from seed-sequence independent effects. Our results suggest that seed sequence independent effects of miR-125a in fact do exist. It is possible that observed decreased red blood cell counts in mice reconstituted with miR-125a or the seed sequence mutant overexpressing HSCs is a result of non-seed region stabilized interactions with certain targets. Moreover, it is likely that transcripts involved in the block of erythroid differentiation are not necessarily expressed in HSCs but in more committed progenitors, therefore CLASH-CLIP and iTRAQ in various hematopoietic populations can provide a better understanding of the mechanism. Further insight into these events could eliminate perturbations in erythroid lineage differentiation that would be undesired outcome of miR-125a overexpression for clinical purposes.
MIR-125 AND ITS NETWORK OF TARGETS AS A NOVEL DIRECTION FOR HSC AND PROGENITOR EXPANSION

The data presented in Chapter 5 provide evidence that miR-125 overexpression can expand HSC and induce stem cell potential in progenitors. As we found this effect occurred in both murine and human cells. This approach would be potentially clinically relevant when aimed to generate stem cells for transplantation purposes. In order to better understand how miR-125 exerts its activity, it would be important to perform in vivo tests to modulate expression of the identified targets, such as p38 and Ptpn1 and analyze to what extent the miR-125 phenotype can be phenocopied by repression of a single or a combination of targets. To not restrict the search to already identified targets in 32D cells, it would be interesting to construct a small molecule library based on results originating from iTRAQ (on control or cells with overexpression or downregulation of miR-125a) and CLASH-CLIP experiments (identifying transcripts interacting with Ago and miR-125a) on different hematopoietic cell populations. Such studies would lead to more detailed analysis of the network of transcripts that interact with miR-125 at different stages of hematopoiesis. The inhibition of targets with small molecules would allow for chemical modification of HSCs and progenitors, without the need of viral transductions, which obviously negatively impacts on potential clinical utility. Moreover, the amplitude of repression may better mimic physiological levels. As a read-out of such a screen liquid cultures, CAFC or CFU-GM assays would suffice for the first test, considering the striking phenotype we observed in in vitro experiments while overexpressing miR-125a in HSPCs.

A parallel line of research could involve testing available libraries of small molecules and quantify their effect on the expression level of endogenous miR-125. As the positive controls molecules such as SR1 (StemRegenin 1 (Boitano et al., 2010)), UM171 (Fares et al., 2014) or PGE2 (Prostaglandin E2, (North et al., 2007)) can be applied, as their potential to expand HSCs was confirmed in vitro. It is conceivable that these molecules affect endogenous miR-125 expression. We speculate that perturbing expression of miR-125a and/or its network of interacting partners in LT-HSCs and progenitors may be an alternative to above mentioned molecules and could improve protocols for HSCs expansion that are currently in clinical testing.

An unresolved issue at current is the cause of mortality observed in mice transplanted with murine cells in which miR-125 was overexpressed, and the absence of such disease when human cells were used. Disease development may be related to the exposure of the transplanted miR-125 overexpressing cells to the cytokine storm and the replicative stress that is inevitable upon lethal irradiation of recipients. Also, the viral integration site, the level of miR-125a overexpression, or potentially collaborating mutations gained during the course of serial transplantation experiments could contribute to disease development. To address these possibilities, integration site analyses of the miR-125a-encoding vector in HSC and progenitors would be recommended, to identify whether sites of integration are located in inter- or intragenic regions, and whether there is an enrichment for specific loci. Secondly, exome sequencing of bone marrow samples from moribund mice could shed a light on collaborating mutations, that occur in serial transplantation experiments. To determine whether the dose of miR-125 is important to induce increased self-renewal, it would be worthwhile to explore transient or inducible expression of this miRNA in hematopoietic stem and progenitor cells. This would also allow to shut down the expression of miR-125 upon reaching certain chimerism levels. It could also resolve the issue of mortality when murine cells are used, as one could test whether these animals would still develop hematological malignancies when miR-125a would be switched off and at the same time if they would have expanded HSC pool.

Collectively, miR-125 could be applicable for clinical use to be included in stem cell expansion protocols. However, potential clinical application will require more effort to better understand the molecular mechanisms by which miR-125a exerts its potent activity, and to separate beneficial stem cell expansion activity from detrimental leukemogenic effects.
REFERENCES