TITLE: An Analysis of microRNA Expression in the Myelodysplastic Syndromes Using Hematopoietic Stem Cells

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We utilized primary bone marrow specimens from patients with the myelodysplastic syndromes (MDS) to identify microRNAs (miRNAs) dysregulated in MDS hematopoietic stem cells (MDS HSCs) as compared with normal HSCs. MiRNAs differentially expressed between MDS HSCs and normal HSCs overlapped significantly with those differentially expressed between HSCs from old and young normal patients, suggesting that MDS pathogenesis may overlap significantly with that of normal aging. A miRNA significantly downregulated in normal older HSCs that was further downregulated in MDS was miR-125b. We examined old and young mice to confirm that miR-125b is also downregulated in older mice, and studies reintroducing miR-125b into old HSCs restored their long-term reconstitution capacity and self-renewal as demonstrated by serial transplantation assays. Together, these findings validate the functional role of miR-125b in hematopoiesis in the context of aging and its likely implication in the age-related predisposition for the development of MDS.
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**Introduction**

Our lab and others have accumulated evidence that the myelodysplastic syndromes are a disorder arising in hematopoietic stem cells (HSCs), demonstrating the presence of disease associated cytogenetic and molecular genetic abnormalities and distinct gene expression programs in purified HSCs from MDS patients. As such, identifying the molecular pathways that regulate MDS HSC function will be critical to developing more effective therapies for MDS. Any curative therapy must eliminate the MDS HSC clone, as this is likely the only self-renewing population in the MDS bone marrow. MicroRNAs (miRNAs) are small non-protein-coding RNAs that effect inhibition of translation and/or degradation of target mRNAs, and miRNAs have been implicated in many different solid and hematologic malignancies. Several studies have evaluated miRNA expression in MDS and observed a distinct signature. A major limitation of these studies has been the use of total bone marrow cell populations, of which phenotypically defined HSC (Lin-CD34+CD38-CD90+CD45RA-) comprise an exceedingly small fraction (approximately one in ten thousand). The studies thus far published are therefore unlikely to reflect the more biologically relevant miRNA expression changes we expect to see in HSC. We believe it is critical to evaluate purified HSC from MDS patients for changes in miRNA expression. Such an examination is likely to reveal dysregulation at the earliest stages of hematopoiesis, from which arises the ineffective hematopoiesis that characterizes MDS.

**Keywords**

MicroRNAs, the myelodysplastic syndromes, hematopoietic stem cells, aging, cell surface markers

**Overall Project Summary**

**Objective 1-** Identify miRNAs That Are Overexpressed in MDS HSCs and Prioritize Candidate Targets for Further Investigation

**Results/Progress and Accomplishments**

We FACS-purified hematopoietic stem cells from six low-risk MDS patients (refractory anemia) with normal cytogenetics and six normal healthy controls (age range 20-35) and performed miRNA profiling using a Taqman based qPCR assay. We identified 79 dysregulated miRNAs (Figure 1), including *miR-125b*, which was decreased in MDS HSCs compared with normal HSCs. Given the disparity between the average age of our MDS patients (65 years old) and normal HSC controls, we went on to FACS-purify HSCs from several aged matched older patients, also obtaining miRNA profiles using the same platform. Interestingly, this revealed only 31 dysregulated miRNAs (Figure 2), with 22 commonly dysregulated miRNAs between the MDS HSC->Young HSC and MDS HSC->Old HSC comparisons (Figure 3).
Figure 1- MDS HSCs exhibit dysregulated miRNA expression compared with HSCs from healthy younger adults. A Taqman based microarray was used to assess the expression of 754 miRNAs in hematopoietic stem cells FACS-purified from six MDS patients as compared with six healthy controls (labeled “young,” as they were aged 20-35). 79 dysregulated miRNAs were identified (FDR<0.1).

Figure 2- MDS HSCs exhibit dysregulated miRNA expression compared with HSCs from healthy older adults. A Taqman based microarray was used to assess the expression of 754 miRNAs in hematopoietic stem cells FACS-purified from six MDS patients as compared with three age matched (age range 65-85) healthy controls (labeled “normal”). 31 dysregulated miRNAs were identified (FDR<0.1).

Figure 3- Many miRNAs dysregulated in MDS HSCs are also dysregulated in aged normal HSCs. Many miRNAs (57) found to be differentially expressed between MDS HSCs and young normal HSCs were no longer found to be differentially expressed in a comparison with age matched HSCs from older adults.
Among the miRNAs found to be significantly downregulated in MDS HSCs was miR-125b (mean 171-fold decrease) but not miR-125a. We have previously demonstrated that miR-125b regulates HSC survival through an anti-apoptotic mechanism, leading to a preferential expansion of lymphoid-biased HSC because of their intrinsically higher baseline levels of apoptosis.\(^\text{12}\) We proposed that downregulation of miR-125b in the MDS HSC contributes to the increased apoptosis that characterizes MDS. Furthermore, preferential apoptosis in lymphoid biased HSCs may explain why the cytogenetic abnormalities, tri-lineage dysplasia, and ineffective hematopoiesis that characterize MDS are not generally found in the lymphoid compartment.\(^\text{13}\) MiR-125b appeared to be downregulated with normal aging and even further in MDS HSCs as compared with older aged matched HSCs (Figures 2-3). Given the well-described association of MDS with aging, as well previous studies describing MDS-like features in aged normal HSCs (e.g. myeloid bias, increased apoptosis, decreased functional output)\(^\text{14}\) we hypothesized that downregulation of miR-125b contributes to the aging phenotypes that predispose to ineffective hematopoiesis and MDS.

**Objective 2- Functionally validate the role of miR-125b in the pathogenesis of the myelodysplastic syndromes (MDS).**

**Results/Progress and Accomplishments**

We used a lentiviral system to express an anti-microRNA against miR-125b (Zip-125b) in mouse hematopoietic stem cells and assessed their function in methylcellulose colony forming assays. Antagonism of miR-125b led to an increase in apoptosis and a decrease in cellular output in methylcellulose colony forming assays (Figure 4), both features reminiscent of the increased apoptosis and decreased hematopoietic output observed in human MDS.

**Figure 4-** Mouse hematopoietic stem cells (lin^−^sca-1^−^c-kit^+^CD34^−^CD150^+^) were FACS-purified to >95% purity and lentivirally transduced to overexpress an anti-miRNA against miR-125b (Zip-125b) or control vector before plating into complete methylcellulose (1000 cells) in triplicate. Cells were harvested after eight days and assessed for apoptosis by Annexin V staining (A) as well as absolute cell number (B) using GFP as a marker of transduction. Wright-Giemsa staining of cytospin preparations of cells transduced with control vector (C) or miRZip-125b (D) demonstrated the presence of dysplastic hypolobated granulocytes with Zip-125b expression. *p=0.03, **p=<0.01
To assess the effects of miR-125b knockdown in vivo, we utilized both young and old mice (9-11 weeks and 27 months of age, respectively), as we demonstrated that miR-125b is downregulated with HSC aging, similar to what we found in human HSCs (Figure 5). Thus, we proposed that examining the effects of miR-125b knockdown and overexpression in the context of both young and old mice would be necessary to fully understand the effects of perturbed miR-125b expression on hematopoiesis.

We utilized a lentiviral system to overexpress Zip-125b in mouse HSCs, followed by non-competetive transplantation into lethally irradiated recipient mice. We found that antagonism of miR-125b led to myeloid biased hematopoiesis (Figure 6), as well as a trend towards decreased engraftment, both features reminiscent of MDS HSCs.

**Figure 5**- Mouse hematopoietic stem cells (lin-sca-1- c-kit-CD34-CD150+) were FACS-purified to >95% purity from young (9-11 weeks, n=5) and old (27 weeks, n=5) mice and miRNA profiling was performed using a Taqman based qPCR platform, demonstrating downregulation of miR-125b in old mice.

**Figure 6**- Mouse hematopoietic stem cells (lin-sca-1- c-kit-CD34-CD150+) were FACS-purified to >95% purity from young and old mice, lentivirally transduced to overexpress miR-125b or an anti-microRNA against miR-125b (Zip-125b) followed by transplantation into lethally irradiated recipient mice. Peripheral blood analysis at 10 weeks after transplantation revealed a significant myeloid bias (Gr-Mac1+ and Gr1+Mac1+ cells) with Zip-125b overexpression (transduced cells marked by GFP).
Figure 7- Mouse hematopoietic stem cells (lin−sca-1−c-kit−CD34−CD150+) were FACS-purified to >95% purity from young and old mice, lentivirally transduced to overexpress miR-125b or an anti-microRNA molecule against miR-125b (Zip-125b) followed by transplantation into lethally irradiated recipient mice. Peripheral blood analysis at 67 days after transplantation revealed a significant increase in engraftment (transduced cells marked by GFP) with miR-125b overexpression in both young and old HSCs, as well as a non-significant trend towards decreased engraftment with Zip-125b expression in both young and old HSCs.

Figure 8- Mouse hematopoietic stem cells (lin−sca-1−c-kit−CD34−CD150+) were FACS-purified to >95% purity from young and old mice, lentivirally transduced to overexpress miR-125b followed by transplantation into lethally irradiated recipient mice. Peripheral blood analysis at the indicated time points after transplantation revealed a significant increase in engraftment (transduced cells marked by GFP) with miR-125b overexpression in both young and old HSCs, as compared with old HSCs transduced with vector control.
In both mice and humans, aged HSCs have decreased engraftment potential in long term reconstitution assays, as well as a myeloid bias, both features that likely pre-dispose older adults to the development of MDS. We thus assessed the effects of miR-125b overexpression in HSCs from old mice. We found that overexpression of miR-125b was able to restore the engraftment potential of old HSCs to a level comparable to that of young HSCs. We furthermore found that overexpression of miR-125b in both young and old HSCs led to an expansion of HSCs (Figure 9). Importantly, the myeloid biased output characteristic of old HSCs appeared to be reversed by miR-125b overexpression (Figure 10), a finding that persisted through secondary transplantation of old HSCs overexpressing miR-125b.

**Figure 9-** Mouse hematopoietic stem cells (lin-sca-1-c-kit+CD34-CD150+) were FACS-purified to >95% purity from young and old mice, lentivirally transduced to overexpress miR-125b followed by transplantation into lethally irradiated recipient mice. Analysis of the bone marrow twelve weeks after transplantation revealed an expansion in both total HSCs and lymphoid biased HSCs in both young and old HSCs transduced to overexpress miR-125b (top panel). The majority of the composition of each hematopoietic stem and progenitor cell type was derived from miR-125b transduced cells (marked by GFP) (bottom panel).
Together, these findings validate the functional role of miR-125b in hematopoiesis in the context of aging and its likely implication in the age-related predisposition for the development of MDS. The observation that miR-125b knockdown induces dysplastic maturation and increases apoptosis in vitro, as well as decreased engraftment and myeloid biased maturation in vivo, strongly suggests that miR-125b contributes to the development of these same features in MDS. The fact that miR-125b overexpression can reverse the impaired engraftment potential and myeloid bias of aged HSCs also suggests that it may be able to do the same in MDS HSCs.

To determine whether miR-125b can restore normal hematopoietic function in the context of MDS, we are currently performing experiments to overexpress miR-125b in primary MDS HSCs to assess whether we can improve their functional output. These experiments have been difficult to perform due to the limiting numbers of HSCs we have been able to obtain from primary MDS specimens (average ~300-500 HSCs), as well as technical challenges in efficient lentiviral transduction of primary human cells. We have been addressing these concerns by working with our clinical collaborators to obtain high cellularity bone marrow...
specimens and optimizing our lentiviral transduction protocols, and we anticipate we will be able to complete these aims in the next six to twelve months.

**Key Research Accomplishments**

- We performed the first comprehensive profiling of miRNA expression in purified hematopoietic stem cells from patients with MDS.
- We identified commonly dysregulated miRNAs in HSCs in the context of normal aging and MDS.
- We demonstrated that \textit{miR-125b} is downregulated with normal aging and further downregulated in MDS.
- We demonstrated that antagonism of \textit{miR-125b} in normal HSCs promotes features reminiscent of MDS, validating the likely functional relevance of this miRNA in MDS pathogenesis.
- We demonstrated that \textit{miR-125b} overexpression reverses functional deficits displayed by aged HSCs that likely contribute to the development of MDS.

**Conclusions**

By examining miRNA expression in purified disease-initiating hematopoietic stem cells in MDS, we revealed molecular features common to normal aging and MDS. By studying a miRNA downregulated in both contexts, we identified the potential for this miRNA to reverse the decreased functional output and myeloid bias characteristic of both MDS and aging. In ongoing experiments we are attempting to overexpress \textit{miR-125b} in primary MDS HSCs to assess whether we can improve their functional output. Finally, to identify functionally relevant transcripts targeted by \textit{miR-125b}, we are performing an integrated transcriptomal and miRNA analysis generated from the same samples. Based on this analysis we aim to identify relevant targets that might be inhibited to recapitulate the effects of \textit{miR-125b} overexpression. Such a therapeutic strategy would have the potential to improve the function of HSCs in the context of both MDS and aging.

**Publications, Abstracts, and Presentations**

**Peer-Reviewed Scientific Journals:**


**Invited Articles:**

Inventions, Patents, and Licenses:
None

Reportable Outcomes:
• Identification of miR-125b downregulation as a key pathogenic feature of the development of MDS and normal aging.

Other Achievements:
During the time frame of support from this career development award, I have made other contributions to our understanding of disease initiating stem cells in hematologic malignancies. Specifically, my work has led to the identification of CD99 as a novel disease stem cell marker and therapeutic target on MDS and AML stem cells, as well as the identification of HSCs as the disease initiating stem cells in hairy cell leukemia. This work has led to the below listed peer-reviewed manuscripts and presentations at international meetings:

Peer-Reviewed Scientific Journals:
*cover article

Abstracts/Oral Presentations:
1. American Society of Hematology, Annual Meeting, 2012:
2. International Society of Experimental Hematology, Annual Meeting 2013:
3. American Society of Hematology, Annual Meeting, 2013:
4. International Society of Experimental Hematology, Annual Meeting, 2014:
   Chung SS, Devlin S, Klimek VK, Park CY. CD99 is a Therapeutic Target on Disease Stem Cells in the Myelodysplastic Syndromes and Acute Myeloid Leukemia. Session 6: The Myelodysplastic Syndromes.
Opportunities for Training and Professional Development:
During the time frame of this award I have had the opportunity to attend and participate in a number of workshops to develop my expertise in the field of hematopoiesis. This includes attendance at Keystone Symposia (Hematopoiesis), a CFU Assay Training Program (Stem Cell Technologies), as well as attendance at the International Society of Experimental Hematology and American Society of Hematology Annual meetings. I have also been successful in competing for other career development funding, including a Fellow Scholar Award from the American Society of Hematology ($100,000 direct costs, 7/2015-6/2017), which will allow me to continue work on this project with protected research time.

References: