Chapter 4

SPECIFIC ERADICATION OF LEUKEMIC STEM CELLS: CAN MICRORNAS MAKE THE DIFFERENCE?

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BACKGROUND

The treatment and outcome of acute myeloid leukemia (AML) patients depends on several factors, including karyotype and molecular alterations present in the leukemic cells. For over 40 years the standard treatment for AML is a combination of cytostatic drugs consisting of cytarabine and an antracycline such as daunorubicin \(^1\). This combination chemotherapy treatment results in a complete remission (CR) in the majority of patients \(^2\). Unfortunately, despite high remission rates only 30-40% (<60 years) and 10-20% (>60 years) of AML patients survive five years after diagnosis. This dismal outcome is mainly due to recurrence of the disease, caused by a subpopulation of chemotherapy resistant leukemic cells with stem cell-like properties, often referred to as “leukemic stem cells” (LSC) \(^3\)-\(^5\). The clinical importance of LSC is shown by the inverse relationship of their frequency at diagnosis and after chemotherapy with outcome \(^3\),\(^6\).

Once AML has relapsed treatment is difficult and mostly unsuccessful. To improve AML outcome it will be crucial to overcome chemotherapy resistance and eradicate LSC to finally prevent relapse. LSC co-exist with residual healthy hematopoietic stem cells (HSC) in the bone marrow of the AML patient. Increasing the dose of chemotherapy might eliminate these LSC, however will also result in non-specific elimination of HSC, leading to prolonged or permanent marrow aplasia and other off-target toxicities. To increase the survival chances for AML patients, it will be crucial to develop alternative targeted therapies against LSC that spare healthy HSC (Figure 1).

Gene expression profiling (GEP) has been applied to unravel transcriptional programs of normal bone marrow and malignant cells, to identify AML subtypes, stratify patient into responders and non-responders and to predict survival \(^7\)-\(^10\). However, GEP has not been successful in the discovery of genes that upon targeting specifically eradicate leukemia cells. The reason for this might be that complex phenomena like therapy resistance and cancer cell maintenance cannot easily be overruled by targeting a single gene. To unravel resistance mechanisms that might be targetable, GEP studies have been performed on CD34+CD38- LSC that were compared with the total population of AML cells or the subpopulation of leukemic progenitors cells (CD34+CD38+) \(^11\),\(^12\). Additionally, studies have been performed that attempt to identify therapeutic targets that specifically eliminate LSC while sparing healthy HSC by comparing the GEP of LSC with those of HSC \(^13\)-\(^15\). As all past studies have compared AML stem cells with HSC derived from healthy individuals the identified differences may not include changes induced by the leukemic microenvironment. This likely omits the most important LSC survival mechanisms since it has been shown that survival of LSC after chemotherapeutic treatment is highly dependent on the leukemic microenvironment \(^16\). Moreover, the presence of leukemic cells with enhanced expression of LSC and/or HSC gene signatures is associated with a poor patients outcome \(^17\), suggesting that leukemic cells acquire stem cell features and therewith acquire self-renewal capacity and therapy resistance. Since LSC are highly dependent for their survival on the heterogeneous diseased microenvironment \(^4\),\(^16\) and
utilize a variety of mechanisms to resist chemotherapy, the major challenge in targeting all the leukemic cells that contain stem cell properties is their heterogeneity. This review will further focus on the role of miRNAs in LSC and their potential as therapeutic targets.

Figure 1. The role of leukemic stem cells in the origin of relapse. At diagnosis, AML consist of a heterogeneous population of leukemic (stem) cells and residual normal hematopoietic (stem) cells. Conventional chemotherapy often results in complete remission, frequently with the persistence of a small number of leukemic cells that survived treatment. This population is enriched for chemotherapy resistant LSC that have the ability to self-renew and induced a relapse. Alternative targeted therapies (e.g. small molecules, monoclonal antibodies and/or miRNA-based strategies) that specifically target the LSC while sparing, or ultimately stimulating, HSC are needed to prevent relapse without significant toxicity.

MiRNAs are a class of small, non-coding RNAs of 18-25 nucleotides that post-transcriptionally control the translation and stability of mRNAs. The number of known miRNAs is growing rapidly, and more than 700 miRNA genes have already been identified in the human genome alone, which is approximately 3% of the known human genes (Sanger miRBase). The biosynthesis of miRNAs starts with a nuclear precursor miRNA produced by Drosha which is further processed in the cytoplasm by the protein Dicer to a mature functional miRNA. By targeting tens to hundreds of genes, miRNAs can direct basic biological functions and pathways but also contribute to cancer development and progression. Examples are their involvement in the regulation of the maintenance and function of differentiation and development of mature progeny from HSC and the involvement in leukemogenesis. AML is derived from HSC or progenitors that have acquired mutations and the subsequent aberrant expression of miRNAs can be either oncogenic or tumor suppressive in this process. Like other types of cancer, AML is characterized by abnormal miRNA expression patterns that strongly correlate with tumor classification, cytogenetics, molecular abnormalities, prognosis and therapy response. Importantly, miRNA expression signatures can provide prognostic information in addition to cytogenetics, mutation analysis, and gene expression. MiRNA expression profiles
are highly specific and possibly even more potent in disease classification and therapy response and outcome prediction than GEP, likely because of their enhanced stability as compared to mRNAs. Indeed, we identified a miRNA expression profile that could classify acute leukemia of ambiguous lineage as either lymphoid or myeloid while GEP had an unclassifiable subset.

Aberrant expression of miRNAs contribute to the character of the cancer and can be either oncogenic or tumor suppressive depending on the cellular context and the available miRNA targets. Regarding the heterogeneity of AML and consequently LSC leading to a presumed unresponsiveness by targeting a single gene, miRNA modulation may hold the key to successful elimination of therapy resistant leukemic (stem) cells either by inducing apoptosis or by sensitizing to chemotherapy. Moreover, since there is differential expression of miRNAs in AML and normal bone marrow as well as in LSC and HSC and LSC and leukemic progenitors, agents that modulate the activity of miRNAs might potentially lead to leukemia and/or LSC-specific effects.

To elucidate the differential expression of miRNAs in LSC, HSC and leukemic progenitors that all reside within the AML bone marrow we performed miRNA expression profiling. By using the activity of aldehyde dehydrogenases (ALDH) together with previously identified leukemia-associated biomarkers we obtained miRNA expression profiles of LSC, leukemic progenitors and HSC all obtained from the same AML bone marrow sample. This approach resulted in the identification of miRNAs differentially expressed between LSC and HSC and between LSC and leukemic progenitors. Several of those miRNAs showed to be specifically expressed in LSC and/or HSC, have prognostic value in AML and/or showed to have the potential to function as targets for miRNA-based anti-LSC therapy.

In the next part of the manuscript we will focus on the expression and function of the by us identified miRNAs in stem cells in AML. We will also review the recent literature regarding the involvement of these miRNAs in AML pathogenesis and their potential as targets and tools for leukemia- and LSC-specific therapeutics.

MicroRNA expression in healthy HSC

HSC have the ability to undergo self-renewal and give rise to all cells of the hematopoietic system during the human life. To do so, the balance between self-renewal and differentiation is strictly controlled by several molecular factors, including miRNAs. The expression and function of miRNAs during hematopoiesis have been studied intensively over the last years but studies investigating miRNA expression in highly enriched human stem and progenitor cell populations are scarce. This is partly due to a lack of consensus on which markers to use for isolation of highly purified HSC but also to the difficulty in isolating sufficient numbers of HSC. Human HSC reside within the immuno-phenotypical defined compartment of lineage negative (Lin-) CD34+CD38-CD90+CD45RA- cells, which differentiate into Lin-CD34+CD38-CD90-CD45RA- multipotent progenitors (MPP) with both lymphoid and myeloid potential. The more committed Lin-CD34+CD38+...
Figure 2. MicroRNA biogenesis and function. Primary miRNAs (pri-miRNA) are transcribed from miRNA genes by RNA polymerase II (RNA-Pol II) and contain a 7-methylguanosine cap (Cap) and a 3' poly(A) tail. In the nucleus, the endonuclease Drosha together with a double-stranded RNA (dsRNA)-binding protein (DGCR8) cleave the stem–loop structure of the pri-miRNA. This results in a precursor miRNA (pre-miRNA) which is exported from the nucleus by the RanGTP-dependent dsRNA-binding protein exportin 5 (XPO5). In the cytoplasm the pre-miRNA is then further cleaved by the endonuclease Dicer together with its dsRNA-binding partner TRBP (transactivation-response RNA-binding protein) leading to a miRNA–miRNA* duplex. This duplex is loaded into an Argonaute (AGO) protein, a step that is mediated by HSP70/HSP90 which induces a conformational change of the AGO protein enabling binding of the stiff dsRNA. The mature RNA-induced-silencing complex (RISC) is formed when miRNA*-strand is expelled from the AGO protein. The RISC binds to target mRNA resulting in miRNA-mediated post-transcriptional gene silencing via multiple mechanisms. The RISC complex can inhibit the initiation of translation by affecting the recruitment of 40S small ribosomal subunit and/or by inhibiting the 60S subunit and thereby the formation of the 80S ribosomal complex. Alternatively the RISC may obstruct translation after initiation by inhibiting the elongation of ribosomes. Moreover, RISC binding can lead to the recruitment of RNA decapping and/or deadenylating enzymes that lead to mRNA destabilization. Some of the target mRNAs bound by the RISC are transported into cytoplasmic processing bodies (P-bodies) for degradation or storage.

common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP) that develop from MPP, can be separated using the differential expression of CD123, CD110 and CD45RA. In healthy individuals the HSC and all the immature progenitor populations express CD133.
The expression of miRNAs has been mostly studied in fractions containing murine HSC, in human CD34+ and CD133+ cell fractions and in the more HSC-enriched human CD34+CD38- and CD90+CD45RA- fractions. The group of Georgantas performed the first large scale miRNA profiling of human CD34+ peripheral blood and bone marrow cells and identified 33 miRNAs. Since then other groups have performed similar analyses in phenotypically more HSC-enriched fractions. Both miR-29a and miR-125b consistently showed higher expression levels in HSC (Lin−CD34+CD38−CD90+CD45RA−) and multipotent progenitors (Lin−CD34+CD38−CD90−CD45RA−) than in committed, differentiated progenitors. Various miRNA expression studies are characterized by considerable variation in results. This is partly due to the use of different profiling methods but also due to the isolation and profiling of different immune-phenotypically defined HSC compartments and the comparison with a diversity of control populations (e.g. whole bone marrow, total CD34+ population or well defined progenitor populations) which results in variability in the discovered differentially expressed miRNAs. Functional studies linking specific miRNAs to stem cell characteristics like self-renewal, regulation of hematopoietic differentiation and programmed cell death are therefore needed to confirm the importance of their differential expression. Various studies investigating the functional role of specific miRNAs in normal hematopoiesis and leukemogenesis have been published. Many of the miRNAs identified by the expression profiling studies indeed regulated key processes of hematopoietic development. A summary of these miRNAs that function in myelopoiesis and their identified targets are listed in Table 1.

Importantly several of the identified miRNAs known to increase the self-renewal of HSC appeared also to be aberrantly expressed in AML, suggesting that a failure to modulate their expression during normal hematopoietic development might be responsible for the impaired differentiation, apoptosis and/or enhanced self-renewal that characterizes LSC.

Differential expression of microRNAs in LSC and HSC residing within the AML bone marrow

Although CD34+CD38- cells are enriched for LSC, this cell population is still heterogeneous and includes both normal and leukemic cells. CD34+CD38- HSC and LSC share many features and the extent to which they differ will be of importance for the development of therapies that target LSC and spare HSC. MiRNAs differentially expressed between LSC and HSC may provide targets for these specific anti-LSC therapies. As both LSC and HSC are highly dependent for their survival on the leukemic microenvironment searching for differences in HSC and LSC that are both obtained from the AML patients bone marrow will give the highest chance of finding genuine anti-LSC targets.

For identification of differentially expressed miRNAs between HSC and LSC obtained from one bone marrow the unequivocal identification of both is necessary. Often leukemic (stem) cells have aberrant protein expression, i.e. express cell surface markers
showing lineage infidelity. Frequently observed non-myeloid lineage markers often used to distinguish leukemic and normal myeloid cells are for example CD7, CD19, CD11b and CD56. These lineage markers are generally absent on normal HSC while, in part of the AML cases, expressed on leukemic stem and progenitor cells. Also markers specifically expressed on LSC, including CLL-1 and CD123, are used to identify and discriminate them from HSC.

Generally, the expression of an aberrant immune-phenotypic marker is not absolute; i.e. not expressed on all leukemic cells within one patient, but also not present in all AML patients, which makes it difficult to use one particular biomarker to isolate very pure LSC and HSC fractions from the complete AML patient population [reviewed in 105]. This problem is for a great part circumvented by the use of ALDH activity as a functional biomarker since all HSC have high ALDH activity while CD34+CD38- LSC are characterized by a lower activity in most of the AML cases. Using the difference in ALDH activity in combination with aberrant leukemia-associated marker expression we purified CD34+CD38- HSC, CD34+CD38- LSC and CD34+CD38+ leukemic progenitors (LP) and determined for the first time the miRNA expression profiles of stem cell fractions that are all derived from the same AML bone marrow samples. We found several differentially expressed miRNAs between LSC and HSC as well as between LSC and leukemic progenitors [26] (Figure 3).

The success of anti-LSC therapy relies on efficient eradication of LSC with minimal toxicity, e.g. sparing HSC. To this end, we compared the miRNA expression profiles of LSC with that of residual HSC. MiR-551b, miR-10a, miR-151-5p, miR-29b and miR-125b were much higher expressed in HSC as compared to LSC while miR-181b, miR-221, miR-21 and miR-22 were higher expressed in LSC as compared to HSC, indicating their possible oncogenic function (Figure 3) [26].

Other miRNAs that were found to be highly expressed in residual HSC and thus potentially associated with HSC maintenance or self-renewal are miR-29b and miR-125 [26]. In general, both miRNAs are downregulated in AML patient cells as compared to the normal HSC pool [33,39]. However, higher expression of miR-29b or miR-125b in a subpopulation of AML cases or in a subpopulation of leukemic cells within one AML bone marrow might indicate an association with stem cell properties and a stem cell-like epigenetic state. This is also suggested by the fact that overexpression of miR-29b has similar effects as the hypomethylating agents, 5-azacytidine and decitabine [106]. Downregulation of miR-29b is linked to promotion of DNA hypermethylation in AML cells by directly targeting DNMT3A, DNMT3B and SP1 [106,107]. Since we and others found high expression of miR-29b in healthy HSC we hypothesize that there is an association between its expression, “stemness” and a demethylated state. In contrast to this hypothesis, it has been shown that upregulation of miR-29b inhibits cell proliferation, promotes myeloid differentiation and induces apoptosis [108]. The consequences of overexpression of miR-29b have shown to be cell-type specific and may depend on the differentiation and/or transformation
Table 1. miRNAs involved in myelopoiesis that regulate key (stem) cell processes (like; differentiation, self-renewal, apoptosis and proliferation) and their identified targets.

<table>
<thead>
<tr>
<th>Cell</th>
<th>microRNA</th>
<th>Target</th>
<th>Function</th>
<th>Ref.</th>
</tr>
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<tbody>
<tr>
<td>HSC</td>
<td>Let-7</td>
<td>Hmg2a</td>
<td>Decreases self-renewal</td>
<td>40</td>
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<td></td>
<td>miR-124</td>
<td>Tip110</td>
<td>inhibiting differentiation</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>miR-125a</td>
<td>BAK1</td>
<td>inhibiting apoptosis</td>
<td>42</td>
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<tr>
<td></td>
<td>miR-125b</td>
<td>ABTB1/CDC25C/PPP1CA</td>
<td>Increases proliferation</td>
<td>43,44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bmf/KLF13/p53</td>
<td>inhibiting apoptosis</td>
<td>39,45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>STAT3/c-JUN/JUND/LIN28A/CBF8</td>
<td>inhibiting differentiation</td>
<td>43,46–49</td>
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<tr>
<td></td>
<td>miR-126</td>
<td>HOXA9/Pi3K/akt2/CRKII</td>
<td>Decreases self-renewal</td>
<td>50,51</td>
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<td></td>
<td>miR-132</td>
<td>FOXO3</td>
<td>Increases proliferation</td>
<td>52</td>
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<tr>
<td></td>
<td>miR-146a</td>
<td>TRAF6/IRAK1/STAT1</td>
<td>Increases self-renewal</td>
<td>53–55</td>
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<td></td>
<td>miR-17-92 cluster</td>
<td>E2F1/E2F2</td>
<td>Increases proliferation and block differentiation</td>
<td>56,57</td>
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<td></td>
<td></td>
<td>PTEN/Bim</td>
<td>inhibiting apoptosis</td>
<td>58,59</td>
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<tr>
<td></td>
<td>miR-196b</td>
<td>HOXA9/MEIS1/FAS/HOXB8</td>
<td>inhibiting differentiation</td>
<td>60,61</td>
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<td></td>
<td>miR-24</td>
<td>Bim/CASP9</td>
<td>inhibiting apoptosis</td>
<td>62</td>
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<td></td>
<td>miR-29a</td>
<td>Dnmt3a</td>
<td>Increases self-renewal</td>
<td>63</td>
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<tr>
<td></td>
<td>miR-33</td>
<td>p53</td>
<td>Increases self-renewal</td>
<td>64</td>
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<tr>
<td>MPP</td>
<td>miR-17/20/93/106</td>
<td>SQSTM1</td>
<td>Increases differentiation towards myeloid progenitors</td>
<td>37,65</td>
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<td></td>
<td>miR-24</td>
<td>?</td>
<td>Increases differentiation towards myeloid progenitors</td>
<td>62</td>
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<tr>
<td></td>
<td>miR-29a</td>
<td>HBP1, FZD5, TPM1</td>
<td>Increases differentiation towards myeloid progenitors</td>
<td>38</td>
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<td></td>
<td>miR-520h</td>
<td>ABCG2</td>
<td>Increases differentiation towards myeloid progenitors</td>
<td>32</td>
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<tr>
<td>CMP</td>
<td>miR-142-3p</td>
<td>CCNT2/TAB2</td>
<td>Stimulates granulocytic-macrophage differentiation</td>
<td>66</td>
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<tr>
<td></td>
<td>miR-146b</td>
<td>PDGFRA</td>
<td>Stimulates erythrocytic-megakaryocytic differentiation</td>
<td>67</td>
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<td></td>
<td>miR-155</td>
<td>PU.1</td>
<td>Stimulates granulocytic-macrophage differentiation</td>
<td>68</td>
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<tr>
<td></td>
<td>miR-29a</td>
<td>CCNT2/CDK6</td>
<td>Stimulates granulocytic-macrophage differentiation</td>
<td>66</td>
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<tr>
<td>GMP</td>
<td>miR-130a</td>
<td>C/EBP ε</td>
<td>Inhibits granulocytic differentiation</td>
<td>69</td>
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<td></td>
<td>miR-17-5p/20a/106a</td>
<td>RUNX1</td>
<td>Inhibition of monocytic differentiation and maturation</td>
<td>70</td>
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<td></td>
<td>miR-223</td>
<td>MEF2C</td>
<td>Inhibits progenitor proliferation and granulocyte differentiation</td>
<td>71</td>
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<tr>
<td></td>
<td>NFI-A/E2F1</td>
<td></td>
<td>Stimulates granulocytic differentiation</td>
<td>72–74</td>
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Table 1. (continued)

<table>
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<th>Function</th>
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<td>MEP</td>
<td>miR-105</td>
<td>MYB</td>
<td>Enhances megakaryopoiesis</td>
<td>80</td>
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<tr>
<td>MEP</td>
<td>miR-125b</td>
<td>?</td>
<td>Increasing proliferation and self-renewal</td>
<td>81</td>
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<tr>
<td>MEP</td>
<td>miR-126</td>
<td>MYB</td>
<td>Skews from erythropoiesis towards megakaryopoiesis</td>
<td>82</td>
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<td>MEP</td>
<td>miR-145</td>
<td>Fli-1</td>
<td>Skews from megakaryopoiesis towards erythropoiesis</td>
<td>83</td>
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<tr>
<td>MEP</td>
<td>miR-146a</td>
<td>CXCR4</td>
<td>Impairs megakaryocytic proliferation, differentiation and maturation</td>
<td>84</td>
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<td>miR-15</td>
<td>MYB</td>
<td>Inhibits erythropoiesis</td>
<td>85</td>
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<td>MEP</td>
<td>miR-150</td>
<td>MYB</td>
<td>Skews from erythropoiesi to megakaryopoiesis</td>
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<td>MEP</td>
<td>miR-155</td>
<td>ETS-1/MEIS1</td>
<td>Inhibits megakaryocytic proliferation and differentiation</td>
<td>88</td>
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<tr>
<td>MEP</td>
<td>miR-199b-5p</td>
<td>c-Kit</td>
<td>Promotes erythroid differentiation</td>
<td>89</td>
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<tr>
<td>MEP</td>
<td>miR-221/222</td>
<td>c-Kit</td>
<td>Impairs proliferation and accelerates differentiation of erythroid cells</td>
<td>19</td>
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<tr>
<td>MEP</td>
<td>miR-223</td>
<td>LMO2</td>
<td>Skews from erythroid towards megakaryocytic differentiation</td>
<td>90</td>
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<tr>
<td>MEP</td>
<td>miR-23</td>
<td>SHP2</td>
<td>Promotes erythroid differentiation</td>
<td>91</td>
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<td>MEP</td>
<td>miR-27a/24</td>
<td>GATA2</td>
<td>Promotes erythroid differentiation</td>
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<tr>
<td>MEP</td>
<td>miR-299-5p</td>
<td>?</td>
<td>Skews from erythroid-monocytic towards megakaryocytic-granulocytic differentiation</td>
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<td>MEP</td>
<td>miR-34a</td>
<td>MYB/CDK4/CDK6</td>
<td>Stimulates megakaryocytic differentiation and inhibit cell cycle</td>
<td>94</td>
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<td>MEP</td>
<td>miR-376a</td>
<td>CDK2</td>
<td>Inhibits erythroid differentiation</td>
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<tr>
<td>MEP</td>
<td>miR-451/144</td>
<td>GATA2</td>
<td>Promotes erythroid differentiation</td>
<td>96–98</td>
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<tr>
<td>MEP</td>
<td>miR-486-3p</td>
<td>MAF/BCL11A</td>
<td>Skews from megakaryopoiesis towards erythropoiesis</td>
<td>79,99</td>
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state of the cancer cell. The effect of enhanced miR-29b on the quiescence state of LSC and thereby their chemotherapy sensitivity and survival after treatment has not been extensively investigated in the past.

An expression profiling and functional study by O’Connell et al., identified 11 miRNAs that were enriched in HSC. Ectopic expression of these miRNAs in normal bone marrow demonstrated that miR-125 induces the greatest increase in repopulation potential 33.
MiR-125b overexpression reduced the levels of apoptosis in HSC, likely through inhibition of the pro-apoptotic genes Klf3 and Bmf. Altogether, these results suggest that miR-125b promotes HSC self-renewal. In AML, miR-125b is strongly upregulated as compared to normal bone marrow, particularly in patients with a t(2;11)(p21;q23).

The enhanced expression of miR-125b in myelodysplastic syndrome (MDS) and in AML with a (2;11)(p21;q23) causes a differentiation arrest, suggesting a connection between high miR-125b expression and an immature leukemic phenotype. Moreover, miR-125b overexpression led to a myeloproliferative disorder that progressed to an aggressive form of AML within 3-4 months but was also able to expand the normal HSC pool. Mice transplanted with hematopoietic progenitors overexpressing miR-125b lead to various types of leukemia, including B-cell acute lymphoblastic leukemia, T-cell acute lymphoblastic leukemia or a myeloproliferative neoplasm depending on the degree of miR-125b expression. Together, these functional studies revealed an important role for miR-125 in HSC regulation and leukemogenesis.

We found miR-181b as the most upregulated miRNA in LSC as compared to HSC. MiR-181b is part of a miRNA signature expressed in cytogenetically normal (CN) AML containing high-risk molecular characteristics (e.g. NPM1 negative, FLT3-ITD positive or both) and is associated with a good prognosis. Moreover, a 15 miRNA probe signature, including miR-181b, showed an association with the presence of the CEBPa mutation, possibly explaining the good prognostic characteristics of AML with high expression of the miR-181 family. The overexpression of miR-181b promotes apoptosis and inhibits the viability of MLL-rearranged AML cells.

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Figure 3. Differential expressed miRNAs between normal and leukemic stem cells and between leukemic stem and progenitor cells obtained from single bone marrow AML samples.
We and others found miR-221 and miR-21 highly expressed in LSC and the bulk of the AML as compared to HSC and healthy bone marrow cells. MiR-221 showed to be a good biomarker distinguishing AML from acute lymphoid leukemia (ALL). We observed that mir-21 is highly expressed in LSC and in the AML bulk of most AML patients while normal HSC have much lower expression. This makes, in combination with the fact that miR-21 knockdown in myeloid leukemia cell lines leads to sensitization to various chemotherapeutic agents, targeting of miR-21 a potential therapeutic approach to sensitize LSC for chemotherapy and having limited effect on HSC survival.

**Differential expression of microRNAs in LSC and the AML bulk**

Since LSC are more chemotherapy resistant and are likely to cause relapse, miRNAs that are differentially expressed between these cells and the total leukemic cell burden might be responsible for their survival and re-initiation of disease. We compared the miRNA expression profile of purified LSC and LP populations and identified 12 differentially expressed miRNAs (Figure 3). The top two lower expressed miRNAs in CD34+CD38-LSC as compared to LP are the novel miR-1274a and miR-886. There are not many reports on the function of these miRNAs. However, miR-1274a has recently suggested to be an oncogene involved in cell proliferation and migration of gastric cancer cells. MiR-886 has tumor suppressor activity and shown to target Protein Kinase R (PKR). Low expression of miR-886 has been associated with a poor prognosis in AML, low risk MDS and small cell lung cancer, suggesting that the differential expression of miR-886 in LSC and LP could, at least in part, be responsible for the difference in chemotherapy sensitivity. Increasing the expression of miR-886 might be a potential therapeutic strategy to increase LSC chemotherapy sensitivity.

MiR-126 and miR-126*, miR-22, miR-335 and miR-150 have enhanced expression in LSC as compared to the LP. Interestingly, besides its differential expression between LSC and LP, miR-126 is even more enhanced in HSC. Therefore, we hypothesized that miR-126 might influence stem cell properties and play a role in maintaining the stem cell state in both normal and leukemic stem cells. In line with this hypothesis, Lechman et al. showed that upon knockdown of miR-126 in cord blood hematopoietic stem/progenitor cells there is enhanced proliferation and expansion of the most primitive cells without disturbance of myeloid differentiation. Also knockdown of miR-126 in vivo augmented the frequency of functional CD34+CD38-CD90+CD45RA- stem cells by increasing cell-cycle progression without exhaustion or transformation to malignancy. On the other hand, overexpression of miR-126 led to a progressive reduction of the progenitor supply hampering hematopoietic output. In leukemic stem cells however, knockdown of miR-126 induced apoptosis and differentiation, reducing specifically the clonogenic capacity of LSC and LP without affecting normal HSC. Similar as Lechman et al. we also observed expansion of CD34+CD38- normal HSC after downregulation of miR-126. Subsequently, the group of Dorrance et al. showed that the targeting of miR-126 using nanoparticles, led to a depletion of LSC in an AML xenotransplantation model.
model leading to improved survival of secondary transplanted mice. Moreover, others demonstrated that miR-126 knockdown enhances the responsiveness of leukemic cells to standard chemotherapy. Mechanistically, miR-126 has been shown to target multiple genes affecting the PI3K/AKT/mTOR pathway. In normal HSC higher expression of miR-126 leads to reduced AKT signaling that impaired cell-cycle entry resulting in increased quiescence and a gradual loss of hematopoietic output whereas lower expression of miR-126 and increased AKT signaling results in expansion long-term repopulating HSC. In LSC higher miR-126 expression and thereby lower AKT signaling inhibits cell cycle entry and induces self-renewal via the suppression of CDK3. Conversely, reduced expression of miR-126 in LSC increases AKT signaling, de-repressing CDK3, thereby inducing differentiation and proliferation leading to chemo-sensitivity. The fact that miR-126 regulates the balance between self-renewal and differentiation in AML is in line our findings and those of Li et. al. who showed that miR-126 overexpression activated genes that are highly expressed normal and malignant stem and progenitor cells whereas knockdown induced genes that are enhanced in committed, more differentiated, hematopoietic progenitor cells. A similar association is present in AML. Patients with high miR-126 levels co-express genes that are also present in published stem cell signatures suggesting that these leukemias have a stem cell-like phenotype and possibly similar characteristics like therapy resistance. The observation that AML patients with high miR-126 expression have a poorer survival and higher chance of relapse, could be a result of this. The distinct function of miR-126 in HSC and LSC make it an ideal target for LSC specific miRNA targeting without harming HSC function and potentially even enhancing hematopoietic recovery.

**Figure 4.** The discordant role of miR-126 expression and modulation in HSC and LSC.
MiR-22 is one of the most promising therapeutic targets we found in our expression profile since it is highly expressed in LSC compared to the LP and AML blasts but it has decreased expression in HSC. MiR-22 is found to be overexpressed in MDS and leukemia and associated with poor survival. Mice conditionally expressing miR-22 in the hematopoietic compartment show decreased levels of 5-hydroxymethylcytosine (5-hmC), high HSC self-renewal and develop MDS and hematological malignancies. Functionally miR-22 was shown to target TET2, a member of the TET methylcytosine dioxygenase family, that has been implicated in epigenetic re-programming, embryonic stem cell maintenance and early development, leading to these global changes in 5-hmC levels. As miR-22 is lowly expressed in residual HSC in AML, targeting miR-22 might be effective in hindering a functional property involved in LSC persistence and overcoming therapy resistance without affecting HSC survival.

**Therapeutic approaches to specifically eliminate LSC; sensitization to chemotherapy**

Depending on the expression and function of miRNAs in AML, there are two approaches to develop miRNA-based therapies: antagonists and mimics. Antagonists are made to inhibit miRNAs and are often single-stranded oligonucleotides. Efficient silencing of miRNA activity in vivo requires the chemical modification of these oligonucleotides to improve their binding affinity, biostability and pharmacokinetic properties. The most common modifications to increase the duplex melting temperature and to improve their resistance to nucleases include 2′-O-methyl (2′-O-Me), 2′-Methoxyethyl (2′-MOE) 2′fluoro and the bicyclic locked nucleic acid (LNA) modifications. Among these modifications, LNA exhibits the highest affinity toward complementary RNA. Increased nuclease resistance is achieved by substituting the phosphodiester (PO) backbone linkages with phosphorothioate (PS) which, apart from nuclease resistance, enhances the binding to plasma proteins leading to reduced clearance by glomerular filtration and urinary excretion. Moreover, inhibition of miRNAs by ultra-short 8-mer LNAs, which enable antagonism of a complete miRNA family, can result in therapeutic benefit in mouse disease models. Currently, the most advanced therapeutic miRNA antagonist is directed against miR-122. This LNA has already been successfully tested in Phase II clinical trials for patients with hepatitis C.

Mimics, double stranded oligonucleotides, are used to restore miRNA function and have been used to restore the function of various tumor-suppressive miRNAs. The use of lenti-, adeno- or adeno-associated viruses to drive the expression of a miRNA has been successful applied to reduce tumor growth in mouse models. Clinical application of mimics has started with MRX34, a miRNA mimic of miR-34, which is tested in a phase-I trial that includes also patients with hematological malignancies.

An novel way to make use of the differential expression of a miRNA in LSC and HSC is the use of a lentiviral construct that expresses a suicide gene behind miRNA target sequences. This makes the expression of the suicide gene dependent on presence and
expression level of the chosen miRNA. MiRNAs highly expressed in HSC could potentially inhibit the expression of such a suicide gene whereas leukemic cells with lower miRNA expression lack this escape mechanism resulting specific eradication. This technology has been shown to work in an in vitro and in vivo model whereby expression of a gene, mutated in a lysosomal storage disorder, needed specific expression in differentiated hematopoietic cells as it results in toxicity when expressed in HSC. In this study, miR-126 showed to be the most potent miRNA in the CD34+CD38- cord blood fraction, inhibiting the expression of the enzyme galactocerebrosidase in HSC, while its activity decreases in myeloid differentiation causing expression of galactocerebrosidase and restoring lysosomal storage.

MiRNA-based therapy might not only function as a single agent but also holds great potential in complementing currently used chemotherapeutics. Since a single miRNA can induce global changes in overall gene expression, modulation of miRNA expression might be very effective in targeting a multi-factorial phenomenon like drug resistance. MiRNA modulation has been shown to have the capacity to enhance the response and suppress the resistance to cytotoxic therapies.

The delivery of miRNA modulators to AML LSC within the leukemic bone marrow

Successful delivery of therapeutic miRNA(s) to the leukemic cells in the AML bone marrow, without inducing toxicity, is the final challenge. The charged miRNAs have a small size and low molecular weight making it possible to formulate them into effective delivery systems which reduce their clearance and degradation in the blood. Examples of delivery systems for mimics and anti-miRs are lipids, polyethylenimine, dendrimers, poly(lactide-co-glycolide) particles but also naturally occurring polymers, such as chitosan, protamine and atelocollagen. Importantly, the first liposome-formulated mimic is currently being tested in a Phase I clinical trial in patients with unresectable primary liver cancer. Beside the delivery of mimics and anti-miRs by formulation, viral constructs can be used.

In a study testing the delivery of miR-29b loaded transferrin-conjugated nanoparticles (Tf-NP-miR-29b) to leukemic cells in a xenograft mouse model high uptake and strong downregulation of miR-29b targets in the leukemic cells could be achieved. Tf-NP-miR-29b suppressed AML growth, impaired colony formation, and reduced cell viability in AML patient samples. In addition, Tf-NP-miR-29b also reduced spleen weight and increased overall survival in NSG mice transplanted with AML cell lines. Next to miR-29b, transferrin conjugated nanoparticles containing miR-126 have recently been used. As previously mentioned, treatment with Tf-NP-miR-126 specifically targets the LSC leading to diminished engraftment of both human and mouse AML in secondary recipient transplantations. Together these studies show the great potential for future miRNA based treatment of AML.
CONCLUSION

Two decades ago miRNA research started with the expression profiling of various hematopoietic cell populations and types of leukemia which provided us with an enormous number of miRNAs that could potentially play a regulatory role in normal and malignant hematopoiesis. Indeed many of these miRNAs now have established involvement in controlling differentiation, apoptosis, proliferation and self-renewal in hematopoiesis and leukemia. The most extensively studied miRNAs include miR-125b, miR-29b and miR-126 which are all involved in stem cell regulation and leukemogenesis. MiRNA-based therapy that modulates these miRNAs to prevent leukemogenesis or treat frank leukemia is now possible and holds great potential. Although recent reports on in vivo miRNA treatment are promising, still many issues in optimizing delivery methods and unknown factors like toxicity, due to off-target effects, should be evaluated and solved. It is therefore wanted that future miRNA research focusses on the efficient in vivo delivery and specific targeting of leukemia (stem cells) in order to really bring miRNAs from bench to bedside.
REFERENCES


