Leukemia (2006) 20, 1931–1936

www.nature.com/leu

REVIEW

The role of microRNAs in normal hematopoiesis and hematopoietic malignancies

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Over the past few years, it has become evident that microRNAs (miRNAs) play an important regulatory role in various biological processes. Much effort has been put into the elucidation of their biogenesis, and this has led to the general concept that a number of key regulators are shared with the processing machinery of small interfering RNAs. Despite the recognition that several miRNAs play crucial roles in normal development and in diseases, little is known about their exact molecular function and the identity of their target genes. In this review, we report on the biological relevance of miRNAs for the differentiation of normal hematopoietic cells and on the contribution of deregulated miRNA expression in their malignant counterparts.

Leukemia (2006) **20,** 1931–1936. doi:10.1038/sj.leu.2404387; published online 14 September 2006
Keywords: microRNA; hematopoiesis; lymphoma

Introduction

MicroRNAs (miRNAs) are now recognized as an important class of small RNA molecules that can regulate the expression of many genes. The biogenesis of an miRNA starts with the transcription of a longer primary miRNA transcript (pri-miRNA) by RNA polymerase II (for reviews see Kim, 2005 and Du and Zamore, 2005[1,2]). The pri-miRNA contains a local hairpin structure that is recognized and cleaved by the microprocessor complex. The resulting ~65 nt precursor miRNA (pre-miR) is exported to the cytoplasm for subsequent processing to a ~22 nt miRNA duplex by DICER. Based on the stability at the 5′ends of the miRNA duplex, one strand is loaded in the active RNA-induced silencing complex (RISC). The miRNA will then guide the RISC complex to target mRNA for gene silencing. It is currently thought that in human, the RISC complex acts mainly by mediating specific translation inhibition and to a lesser extent degradation of mRNA targets. Cytoplasmic foci known as processing bodies (P-bodies) are involved in gene silencing mediated by miRNAs.[3] To date, the role of most miRNAs is largely unknown. To obtain better insight in the function of miRNAs, much effort has been put in the computational identification of miRNA targets using various algorithms.[4] However, the drawbacks of these predictions are that they all have a substantial false positive rate and may be biased as they are mostly based on the few known miRNA:target gene interactions. There is significant evidence that miRNAs play an important role in disease, especially in oncogenesis.[5–7] Several miRNAs were shown to be located at fragile sites or at genomic sites that are altered in cancer.[8] Specific miRNAs were shown to be underexpressed and map to regions that are frequently deleted, whereas others were reported to be overexpressed and map at regions that are frequently amplified in cancer.[9–12]

So far, only limited functional data is available on miRNAs in hematopoietic tissues. In contrast, several studies have now reported on their expression patterns (Table 1). miRNA expression was initially determined using a cloning strategy,[13] whereas more commonly applied techniques nowadays include Northern blotting, quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) or microarray technology. Though these latter methods enable a higher throughput, they should be used with caution, especially miRNA microarrays. Irreproducible results may be obtained, for instance due to large differences in individual optimal probe hybridization temperatures. Quantitative RT-PCR specific for mature miRNAs seems to provide more accurate results and is currently considered to be the golden standard.[14] In this review, we provide a comprehensive overview of the expression and function of miRNAs in hematopoietic tissues.

miRNA in hematopoiesis

miR-142s, miR-181a and miR-223

The importance of miRNAs in hematopoiesis was revealed by the identification of three murine hematopoietic tissue-specific expressed miRNAs, miR-181a, miR-142s and miR-223.[13] miR-181a was found to be preferentially expressed in the B-lineage, and ectopic expression of miR-181a in hematopoietic precursor cells resulted in a doubling of B-lineage cells. miR-142s was found to be expressed in B- and myeloid lineages, and miR-223 expression was found to be confined to myeloid lineages. Remarkably, ectopic expression of miR-142s or miR-223 resulted in a 30–50% increase of T-lineage cells and not of B-lineage or myeloid cells.[13] These results show that miRNA can play an important role in the differentiation of hematopoietic cells.

The high expression of miR-223 in the myeloid lineage was confirmed by studying myeloid differentiation in acute promyelocytic leukemia (APL) cells.[15] Stimulation of APL cells with retinoic acid induces differentiation towards granulocytic maturation, which is triggered by the activation of CCAAT/enhancer binding protein α (C/EBPα). The C/EBPα transcription factor competes with the negative regulator nuclear factor IA gene (NFI-A) for binding to the miR-223 promoter and, upon replacement, induces high miR-223 expression. High miR-223 levels result in downregulation of NFI-A protein translation by binding to the 3′-untranslated region of the NFI-A transcript. This indicates that activation of C/EBPα induces granulocytic

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Received 26 April 2006; revised 20 June 2006; accepted 19 July 2006; published online 14 September 2006

Table 1 Overview of microRNA expression and their validated targets in normal hematopoiesis and hematopoietic malignancies

microRNA	*Expression*	*Target*	*Reference*
Normal hematopoiesis			
miR-142	↑B and myeloid lineage[a]		13
miR-155	↑GC B cells and activated T cells		18,20
miR-181a	↑B lineage[a]		13
miR-221	↓Erythropoiesis	KIT receptor	21
miR-222	↓Erythropoiesis	KIT receptor	21
miR-223	↑Myeloid lineage[a], granulopoiesis	NFI-A	13,15
Hematopoietic malignancies			
miR-15a	↓CLL	BCL-2	9,31
miR-16-1	↓CLL	BCL-2	9,31
miR-17-92[b]	↑DLBCL	E2F1	11,51,54
miR-125b-1	Involved in translocation in B-ALL case		37
miR-142	Involved in translocation in B-PLL case		38
miR-155	↑ HL, DLBCL, PMBL, PTLD and CLL, ↓ BL		19,41,45

Abbreviations: B-ALL, B-cell acute lymphoblastic leukemia; BL, Burkitt lymphoma; B-PLL, B-cell prolymphocytic leukemia; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; GC, germinal center; HL, Hodgkin lymphoma; PMBL, primary mediastinal B-cell lymphoma; PTLD, post-transplantation lymphoproliferative disorder.
[a]In murine hematopoietic cells.
[b]The miR-17-92 polycistron.

maturation and that this differentiation is maintained by the induction of miR-223.

Comparison of the miRNA expression profiles of murine hematopoietic cell populations at different stages of differentiation revealed a very strong correlation in cell populations that were at a similar stage of differentiation.[16] The correlation between mature cell populations and their precursors was, however, much lower. This strengthens the concept that miRNAs have an important role in the differentiation and maintenance of specific hematopoietic maturation stages.

miR-155

A few studies reported on the expression of *BIC* or pri-miR-155 in B and T cells and in lymphoid tissues.[17,18] RNA *in situ* hybridization revealed a limited number of *BIC*-positive B cells located predominantly within the germinal centers (GCs) of lymphoid tissues. Northern blotting confirmed expression of the *BIC*-derived miR-155 in total tonsil and reactive lymph node tissues.[19] The expression of *BIC* in GC B cells suggested a possible link of *BIC* expression with the B-cell maturation process regulated by B-cell receptor (BcR)–antigen interaction. This was indeed confirmed in the Burkitt lymphoma (BL) cell line Ramos, which is commonly used as a model system to study effects of BcR triggering in GC B cells.[18] These data provide evidence for a role of miR-155 in the antigen-triggered maturation process of naive B cells.

Stimulation of CD4+ T cells using anti-CD3 in combination with anti-CD28 did result in a strong upregulation of *BIC* expression.[20] This induction could be blocked by calcineurin blockers like cyclosporin A or FK506. The data suggest an association between activation of B and T cells and induction of high *BIC*/miR-155 levels. Although several putative miR-155 target genes have been identified, none of these has been experimentally validated.

miR-221 and miR-222

Downregulation of miR-221 and miR-222 in erythropoietic cells was observed at different stages of erythrocyte differentiation and maturation.[21] Sequence comparison of both miRNAs revealed that the first 8 nt of the 5′ sites are identical, whereas the 3′ sites have only limited sequence similarity. The first 8 nt on the 5′ site of an miRNA contains the so-called seed, which is crucial for miRNA:target gene recognition.[4] The fact that these two miRNAs have identical seed sequences indicates overlap in their target gene repertoire. Target gene prediction studies suggested that the KIT receptor is a target for both these miRNAs. KIT is a key factor for the proliferation control of primitive hematopoietic and erythropoietic cells. Expression studies indeed indicated an inverse correlation of KIT and miR-221/miR-222 expression upon erythropocyte differentiation.[21] Luciferase-based assays provided further evidence that KIT is a true target of miR-221 and miR-222. This indicates that downregulation of miR-221 and miR-222 is a crucial event in erythropoiesis by unblocking the translational repression of the KIT receptor.

DICER knockout

Two studies have reported on the general role of miRNAs in murine T-cell differentiation by generation of a conditional DICER-1 knockout allele.[22,23] The difference between both studies was the use of lckCre transgenic or CD4Cre transgenic mice resulting in, respectively, a DICER knock-out phenotype induced from the first stage or at a much later stage of T-cell development. Remarkably, absence of DICER did not result in a block of T-cell differentiation. Nonetheless, several effects of DICER absence were observed. For example, both studies showed a reduced viability of T cells with more severe effects in the CD4Cre transgenic mice. These mice demonstrated a 2- to 4-fold decrease in the amount of CD4+ and CD8+ T cells in spleen and lymph node and displayed aberrant cytokine expression in T-helper cells.[23] Owing to residual DICER activity or miRNA half-lives beyond that of DICER, the observed phenotype may not be as pronounced as it might have been in a complete absence of miRNAs during T-cell differentiation.

miRNA in leukemia

Chronic lymphocytic leukemia

Hemi- or homozygous deletions at 13q14 are frequently observed in chronic lymphocytic leukemia (CLL), suggesting

the presence of one or more tumor suppressor genes in that region.[24–26] Recently, it was shown that miR-15a and miR-16-1 form an miRNA cluster that maps within the 13q14 deletion.[9] Reduced miR-15a and miR-16-1 levels were observed in 75% of CLL cases with a hemi- or homozygous deletion at 13q14 and in 55% of cases without a 13q14 deletion. The low miR-15a and miR-16-1 expression levels support the hypothesis that these two miRNAs represent the actual targets of the deletion. Analysis of the genomic sequences encompassing the miR-15a and miR-16-1 stem loop and flanking sequences in order to explain reduced expression levels in cases without deletions revealed no mutations in the precursor sequences in 144 CLL cases.[27,28] The existence of a highly similar miRNA cluster at 3q25–26.1 containing the miR-15b and miR-16-2 miRNA genes adds complexity.[29,30] miR-15b differs from miR-15a by 4 nt outside the seed sequence and miR-16-2 is identical to miR-16-1. Reduced miR-16 levels may thus depend on reduced expression of both pri-miRNA transcripts. Clearly, further studies are needed to determine the exact role of each miRNA cluster in CLL pathogenesis.

A potential interesting target gene of miR-15a/miR-16-1, but also miR-15b/miR-16-2, is the antiapoptotic gene *BCL-2*.[31] Protein expression of BCL-2 was inversely correlated with the expression of miR-15a and miR-16-1 in 26 CLL cases. In addition, ectopic expression of miR-15a and miR-16-1 in a cell line with high levels of BCL-2 resulted in a strong reduction of BCL-2 protein levels and induction of apoptosis. Reduction of both members of the miR-15 and miR-16 family may thus be considered as a possible therapeutic target to block the BCL-2 oncogenic pathway in CLL.

Two studies report on miRNA expression profiling in CLL cases using microarray technology.[28,32] In the first study, comparison of the miRNA profile of 38 CLL cases with that of normal CD5+ B cells revealed significant differential expression levels for 55/161 miRNAs, whereas miR-15a or miR-16-1 were not differentially expressed.[32] Supervised clustering using IgV_H mutation status, a marker of prognosis, revealed five associated miRNAs. Surprisingly, a similar, more extensive follow-up study from the same group resulted in an almost completely different miRNA signature using a similar clustering strategy.[28] miR-15a and miR-16-1 were part of this signature and were expressed at higher levels in ZAP70+/IgV_H mutation-negative CLL cases. For CLL, several indicators of prognosis are known, including IgV_H mutation status, ZAP70 expression, 13q14 deletion and BCL-2 expression (Table 2). High levels of miR-15a and miR-16-1 in ZAP70+/IgV_H mutation-negative CLL cases would result in low levels of target gene BCL-2. However, low levels of BCL-2 correlate with good prognosis, whereas ZAP70+ and unmutated IgV_H genes are both indicators of a bad prognosis.[33–35] It is also known that 13q14 deletions as the sole aberration in CLL cases are an indicator of good prognosis.[24] These 13q14 deletions result in lowered miR-15a and miR-16-1 levels which in turn would result in high BCL-2 levels, an indicator of bad prognosis.[36] These results are obviously conflicting and need careful further investigation.

miRNAs as translocation partners

Two B-cell leukemia case studies have been reported on the involvement of miRNAs in translocations. In the case of an acute lymphoblastic B-cell leukemia, 11q24 sequences were observed as inserted in the *IgH* gene locus.[37] Sequence analysis of this 213 bp DNA fragment of 11q24 revealed presence of the complete miR-125b-1 sequence. Lack of sufficient patient material precluded analysis of miR-125b-1 expression levels in the tumor. In a patient presenting with aggressive B-cell leukemia, a (8;17) translocation was observed in which exon 1 of the *MYC* gene was replaced by the miR-142 promoter, 5′ portion and the stem-loop sequences (originally termed *BCL-3*).[38] The 3′ portion of miR-142 was replaced by the truncated *MYC* gene. The fusion transcript was highly expressed in the tumor cells, leading to increased MYC protein levels. The contribution of miRNAs as causative partners in chromosomal translocations can, of course, not be made based on these two cases.

miRNAs in lymphoma

miR-155

The *BIC* (B-cell integration cluster) locus was originally identified as a target for proviral insertions in avian leukosis virus (AVL)-induced lymphomas.[39] Analyses of this locus led to the identification of the *BIC* gene that lacked an extensive open reading frame (ORF) and was thought to function as a non-coding RNA.[40] Comparison of the human, mouse and chicken sequences revealed low homology except for a small region within the last exon of the *BIC* transcript that contained an imperfect stem-loop structure and 78% sequence homology over 138 nt.[17] In 2002, Tuschl and co-workers described the cloning of the *BIC*-derived miR-155 sequence and demonstrated that *BIC* is a pri-miRNA.[29] The pre-miRNA sequence was indeed located within the previously identified interspecies conserved stem-loop structure. Ectopic expression of *BIC* in a *BIC* and miR-155-negative cell line resulted in high levels of miR-155 confirming that *BIC* is a pri-miRNA.[41]

The first evidence for an important role of *BIC* in human cancer came from a study showing high expression of *BIC* in the Hodgkin and Reed–Sternberg cells (HRS cells) of Hodgkin's lymphoma (HL).[18] Two additional studies confirmed the presence of high *BIC* expression levels in HRS cells and also demonstrated the abundant expression of miR-155 by Northern blotting.[19,41] RNA *in situ* hybridization experiments using a locked nucleic acid (LNA) antisense[42] miR-155 probe confirmed presence of miR-155 in the cytoplasm of tissue sections of HL (Figure 1). *BIC* and miR-155 are also expressed in primary mediastinal B-cell lymphoma and diffuse large B-cell lymphoma (DLBCL), especially of the activated B-cell type. Interestingly, these two non-Hodgkin's lymphoma (NHL) subtypes demonstrate a marked overlap in their gene expression profiles with classical Hodgkin's lymphoma (cHL).[19,41,43] Several other NHL subtypes, including follicular lymphoma (FL) and BL, were

Table 2 Prognostic factors in chronic lymphocytic leukemia

Prognostic factor	*Good prognosis*	*Bad prognosis*	*miR-15a/miR-16-1*
IgV_H[33,34]	Mutated	Unmutated	High in unmutated IgV_H[28]
ZAP70[35]	Low	High	High in ZAP70+[28]
13q14 deletion[a 24]	Yes	No	Low in 13q14 deletion[9]
BCL-2[36]	Low	High	Inverse correlation[31]

[a]As sole aberration.

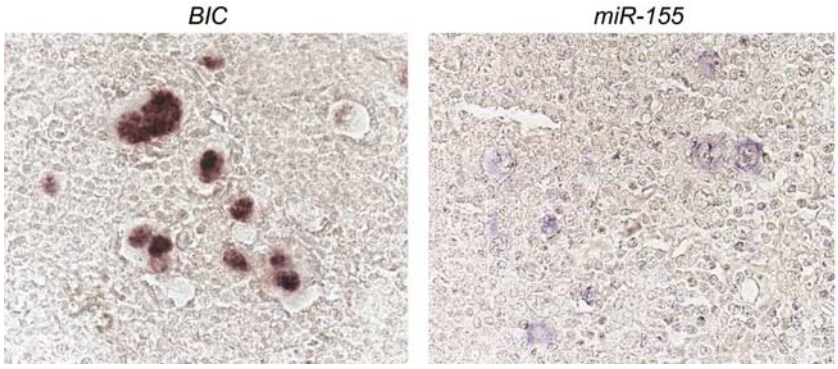

Figure 1 *BIC* and miR-155 RNA *in situ* hybridization in a cHL case. *BIC* staining was performed as described previously.[18] miR-155 staining was performed using a LNA probe antisense to miR-155 (Exiqon, Denmark) according to the manufacturer's protocol. Note the nuclear *BIC* staining and the cytoplasmic miR-155 staining. Original magnification $\times 630$.

negative for *BIC*. A correlation between Epstein–Barr virus (EBV) latency type III infection and miR-155 expression was suggested based on high *BIC* and miR-155 levels in EBV-positive post-transplantation lymphoproliferative disorder and EBV-transformed lymphoblastoid B-cell lines.[44,45]

In the AVL-induced lymphoma model in chicken, integration of proviral sequences in the *BIC* locus was found to be frequently accompanied by insertional activation of the *MYC* locus.[39] A further analysis of a possible cooperation between these two genes was performed by co-expression of MYC and *BIC* in chicken embryo fibroblasts which indeed enhanced growth of these cells.[46] Moreover, co-transfection of MYC and *BIC* in chicken embryos resulted in a lower overall survival and an increased incidence of short-latency lymphomas. Based on these findings, it was suggested that there is oncogenic cooperation between *BIC* and MYC. However, the consistent lack of *BIC* and miR-155 in BL cases with a proven *MYC* translocation, argues against an oncogenic cooperation between MYC protein and *BIC*, at least in human BL.[45]

In a very recent study, miR-155 was placed behind the Eμ enhancer in transgenic mice.[47] These mice showed pre-B-cell like proliferations ($CD45^{low}/CD10^{low}/IgM^{-}/CD5^{-}/TCR^{-}/CD43^{-}$) at 3 weeks of age in both spleen and bone marrow. Later on, these mice developed enlarged spleens, lymphopenia and bone marrow replacement, all features of high-grade B-cell malignancies. However, the B-cell proliferations of the transgenic mice were mostly polyclonal, at least in mice up to 6 weeks of age. Thus, it remains to be seen whether these proliferations are (or will develop into) true B-cell malignancies. Nonetheless, the studies are at least suggestive for an oncogenic role of *BIC*. In light of the previous paragraph, it would be interesting to determine whether the observed proliferations contain *MYC* aberrations. The fact that these mice develop leukemia-like proliferations instead of lymphomas may have to do with usage of the Eμ enhancer. This promoter becomes active at the late pro-B-cell stage. Thus, developing B cells in these transgenic mice will have enhanced miR-155 levels at an early stage. This may not be consistent to the natural situation in which it is thought that B cells express *BIC* upon activation in the GC reaction.[18] It would be interesting to study the phenotype of transgenic mice with miR-155 expression under the control of a GC marker like BCL-6.

Taken together, these studies strongly suggest an oncogenic role for *BIC*/miR-155. Definitive proof on the oncogenic potential of miR-155 may come from further transgenic mice studies and verification of the predicted miR-155 target genes.

miR-17-92 polycistron

Amplification of 13q31 is frequently observed in various NHL subtypes and solid tumors, including DLBCL, FL, primary cutaneous B-cell lymphoma and lung cancer.[48–52] The candidate target gene at 13q31 was *c13orf25*.[50] Similar to the *BIC* gene, transcripts of *c13orf25* were unlikely to encode for a protein as predicted ORFs were short (<70 amino acids) and not conserved in closely related species. Based on miRNA cloning studies,[53] it was noted that transcripts of c13orf25 contain a cluster of seven miRNAs. This cluster was named the miR-17-92 polycistron and included miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1.[11,50,54] miRNA expression profiling and Northern blotting in human B-cell lymphoma cell lines revealed high expression of six members of the miR-17-92 polycistron in cell lines with 13q31 amplifications, with the exception of miR-18a, perhaps due to post-transcriptional regulation of this polycistron.[11,12]

The only proven target of miR-17-5p and miR-20a is the transcription factor E2F1.[54] Expression of this gene is regulated by MYC and it acts as an activator of cell-cycle progression. However, accumulation of E2F1 protein results in apoptosis.[55,56] Ectopic MYC expression in B cells also resulted in increased expression levels of the miR-17-92 polycistron.[54] Chromatin immunoprecipitation confirmed binding of MYC to the upstream genomic region of this miRNA cluster. This clearly indicated that MYC was responsible for the enhanced expression levels of the miR-17-92 cluster. These data suggest a model where MYC not only induces the expression of *E2F1*, but also regulates its protein levels by the simultaneous induction of *c13orf25*. The tight regulation of E2F1 protein by miR-17-5p and miR-20a allows cell-cycle progression and prevents induction of apoptosis.

Another potentially interesting lead for the oncogenic potential of this miRNA cluster came from co-transfection experiments of various randomly selected subsets of miRNA in *Eμ-MYC/+* mice.[11] In this mouse model, MYC expression is driven by the Eμ enhancer which leads to the development of B-cell lymphomas at 4–6 months of age. Transplantation of hematopoietic stem cells (HSC) from *Eμ-MYC/+* mice to lethally irradiated recipients result in similar B-cell lymphoma latencies. Co-expression of the miR-17-92 cluster in *Eμ-MYC/+* HSC and the subsequent transplantation to lethally irradiated recipients resulted in the development of leukemia at an average time interval of 51 days. The observed strongly reduced latency period indicated that the miR-17-92 cluster can accelerate tumor development in cooperation with MYC. In contrast, none

of the other tested miRNA subsets showed signs of accelerated onset of disease. Unfortunately, miR-155 was not studied in this model. At present, no data are available on the expression levels of miR-155 and the miR-17-92 cluster in the MYC-induced lymphomas that develop in the ALV-induced chicken model or in the *Eμ-MYC/+* mice model.

Conclusion/future perspectives

Based on the data presented, miRNAs emerge as essential factors in the regulation of normal hematopoiesis and, as a consequence, deregulation of miRNA expression levels contributes to the development of hematological malignancies (Table 1). Experimental studies indicate that miRNAs can function as tumor suppressor genes or as oncogenes. A loss of function or tumor suppressor property has been demonstrated for miR-15 and miR-16, which induce downregulation of the antiapoptotic BCL-2 protein. A gain of function or oncogenic property has been demonstrated for miR-155 and the miR-17-92 polycistron in MYC induced and transgenic animal models.

To resolve the total impact of miRNA on normal and neoplastic development, reliable *in silico* target prediction algorithms and strategies that allow large-scale screening of multiple target genes are required. Generation of miRNA transgenic or knockout mice will, undoubtedly, provide new insights and interesting leads in the near future.

Acknowledgements

This work was supported by a grant from the Dutch Cancer Society (RUG 01-2414). The authors declare that this paper contains original unpublished work and is not being submitted for publication elsewhere; that there is no financial or other relationships that might lead to a conflict of interest in this document; that this paper has been read and approved by all the authors; that the requirements for authorship have been met and that each author believes that this paper represents honest work.

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