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Mini review

microRNAs: FINE TUNING OF ERYTHROPOIESIS

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Abstract: Cell proliferation and differentiation is a complex process involving many cellular mechanisms. One of the best-studied phenomena in cell differentiation is erythrocyte development during hematopoiesis in vertebrates. In recent years, a new class of small, endogenous, non-coding RNAs called microRNAs (miRNAs) emerged as important regulators of gene expression at the post-transcriptional level. Thousands of miRNAs have been identified in various organisms, including protozoa, fungi, bacteria and viruses, proving that the regulatory miRNA pathway is conserved in evolution. There are many examples of miRNA-mediated regulation of gene expression in the processes of cell proliferation, differentiation and apoptosis, and in cancer genesis. Many of the collected data clearly show the dependence of the proteome of a cell on the qualitative and quantitative composition of endogenous miRNAs. Numerous

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Abbreviations used: AE1 – anion exchanger 1; Ago2 – eukaryotic translation initiation factor 2C, 2; ARE – antioxidant response element; DGCR8 – DiGeorge syndrome critical region gene 8; GATA-1 – GATA-binding protein 1 (globin transcription factor 1); GATA-2 – GATA-binding protein 2; GSH – glutathione; ha-siRNA – heterochromatin-associated small interfering RNA; miRNA – microRNA; nat-siRNA – natural antisense small interfering RNA; ORF – open reading frame; PHZ – phenylhydrazine; piRNA – piwi-interacting RNA; RISC – RNA-induced silencing complex; RNAi – RNA interference; ROS – reactive oxygen species; scn-siRNA – siRNA-like scan (scn) RNA; siRNA – small interfering RNA; ta-siRNA – trans-acting small interfering RNA; TRBP – TAR (HIV-1) RNA-binding protein 2

specific miRNAs are present in the hematopoietic erythroid line. This review attempts to summarize the state of knowledge on the role of miRNAs in the regulation of different stages of erythropoiesis. Original experimental data and results obtained with bioinformatics tools were combined to elucidate the currently known regulatory network of miRNAs that guide the process of differentiation of red blood cells.

Key words: Hematopoiesis, Erythrocyte, Erythroid differentiation, Erythropoiesis, microRNA (miRNA), microRNA expression

INTRODUCTION

miRNAs varying in length from 21 to 26 nucleotides regulate gene expression post-transcriptionally by controlling mRNA translation or stability in the cytosol [1]. miRNA-mediated repression of gene expression occurs via the RNA-induced silencing complex (RISC) with the miRNAs acting as a matrix that guides the complex to the target mRNA.

This regulation can occur thanks to the partial sequence complementarity between miRNA and sequences in the 3' untranslated region of mRNA (3'UTR). However, there are several known examples of miRNAs binding to the open reading frame (ORF) [2] and to 5'UTR [3-5], particularly in plant gene transcripts [6]. In plants, these regulatory molecules require full complementarity to their target sequences in mRNA, while in animals, bulges and loops are not only tolerated, but seem to be the norm. Essentially, the nucleotide sequences in the stems of miRNA hairpins are strongly conserved, while increased variation in the loop sequences is observed. Although miRNAs pair imperfectly with their targets in animals, the essential situation for miRNA/target interaction is a contiguous and perfect base pairing of miRNA nucleotides 2-8, which are assigned to the "seed" sequence.

The first discovered miRNA was *Caenorhabditis elegans* lin-4, which represses the accumulation of lin-14, an essential protein in larval development [7]. Currently, the database for miRNA, miRBase (version 19) hosts records of 21,264 hairpin precursors expressing 25,141 mature miRNA products in 193 species [1]. These numbers include 1,600 human precursors giving 2,042 mature miRNAs products. Not only can one hairpin precursor give two different mature products, but two precursors from various parts of the genome can also produce identical mature products [8], see also [54].

There are currently three known pathways of miRNA biogenesis. The first is called the canonical pathway. miRNAs are transcribed from their respective loci, generally by RNA polymerase II (Pol II), and processed by the RNase type III endonucleases Drosha (RN3) and Dicer. Those miRNAs transcribed as long hairpin precursors (pri-miRNAs), which often contain sequences for several different miRNAs. Drosha and its dsRNA-binding partners process pri-miRNAs to give shorter hairpins, pre-miRNAs. The Drosha partners are DiGeorge syndrome critical region gene 8 (DGCR8) in mammals or the *pasha* protein in

Drosophila melanogaster. The enzyme complex cleaves the pri-miRNAs, leaving a ~2-nt overhang that is recognized by exportin-5, thus allowing the nuclear export of the resulting pre-miRNA. Processing is continued in the cytosol by Dicer complexed with TAR RNA-binding protein (TRBP) [9] to yield an miRNA/miRNA* duplex through cleavage of the unpaired fragment of the hairpin precursor. The duplex is separated and one strand (guide) is usually selected to function as a mature miRNA, while the other strand (passenger) is often degraded (miRNA*). In cases where there is a higher passenger strand content in the cell, the nomenclature miRNA-3p/miRNA-5p is used instead of miRNA/miRNA* (miRNA-3p is the miRNA derived from the 3' arm of the precursor miRNA and miRNA-5p is the miRNA derived from the 5' arm of the precursor miRNA) [10]. The mature miRNA is then loaded into argonaute (AGO) family protein and can associate with the RISC silencing complex, in which it acts as a guide to the target sequence in the mRNA.

The second pathway, originating from intron sequences that correspond precisely to pre-miRNAs was reported in 2007 in two independent papers [11,12]. It is called the mitron pathway. It involves shorter precursors without the lower part of the stem. These precursors have a structure analogous to those generated in the canonical pathway by Drosha/DGCR8 activity [13].

The third pathway was reported in 2010. It bypasses Dicer, instead using AGO2 catalytic activity (cleavage, uridylation and trimming) to generate mature miRNA [14].

GENOMIC LOCALIZATION OF miRNA-ENCODING SEQUENCES INVOLVED IN ERYTHROPOIESIS

The miRNAs acting in erythroid proliferation are encoded by 130 different genes that are located in different parts of the genome (Suppl. Table S1 at http://dx.doi.org/10.2478/s11658-012-0038-z). About half of these genes (61) are clustered (< 10 kbp) with other miRNA genes. Some of them are situated near each other (~100 bp apart) and can be co-transcribed. Interestingly, only single miRNA genes involved in erythropoiesis have been located on chromosomes 6 and 10, while none have been found on the Y chromosome.

miR-17-92 is an miRNA cluster located on human chromosome 13 and mouse chromosome 14. It is highly conserved in all vertebrate clusters and encodes the precursors of six ubiquitously expressed miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1 [15-17]. Two mature products are known for each of those precursors [18, 19]. In humans, the cluster miR-17-92 is homologous to the miR-106a~363 cluster on the X chromosome and the miR-106b~25 cluster on chromosome 7 (5 in mice), but neither of them is transcribed in the erythroid precursor cell line. Down- or upregulation of certain miRNAs is necessary during erythroid development.

According to the available published data and miRBase, there are 19 examples of mature miRNAs that can be products of different hairpin precursors, encoded

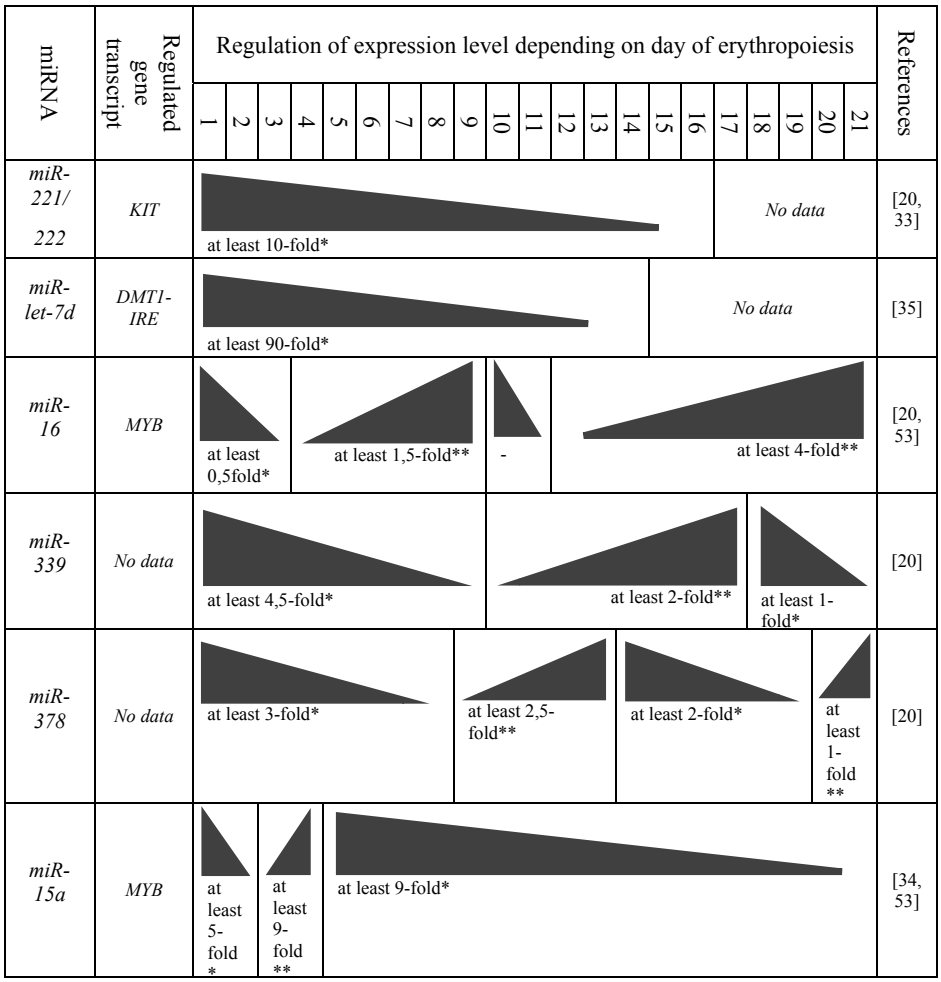
by separate loci in the human genome (Suppl. Table S1). Of them, mature miR-16 can be a product of the miR-16-1 hairpin precursor, encoded by *MIR16-1*, located on chromosome 13q14 or a product of the miR-16-2 hairpin precursor, encoded by *MIR16-2*, located on chromosome 3q25 [46]. Interestingly, hsa-miR-9 can be encoded by genes located on chromosomes 1, 5 and 15. Other examples of mature miRNAs encoded by separate genes are listed in Suppl. Table S1.
There are currently 16 miRNAs with validated target transcripts that are known to play a key role in hematopoiesis. Some of them are variably expressed during the differentiation processes (Table 1) [20]. miRNAs can block erythroblast enucleation [21], inhibit erythropoiesis by downregulating the expression of certain membrane receptors [22], protect against oxidative stress [23] and drive cells toward a specific cell lineage [24].

miR-144/451

miR-144 and miR-451 play an important role in erythropoiesis, as their deregulation affects terminal erythropoiesis [25]. These two miRNAs are transcribed from a single locus on *Danio rerio* chromosome 5 and mouse chromosome 11. Research on *D. rerio* provides strong evidence that this cluster is regulated by GATA-1 transcription factor. *D. rerio* embryos that are depleted of miR-451 develop severe anemia, having reduced levels of hemoglobinized cells. The loss of GATA-1 caused similar but more severe effects: after 24 h of red blood cell development, a reduction in the level of the erythroid markers βe1-globin and AE1 was also observed in miR-451-silenced embryos. In addition, in murine cells depleted of GATA-1, overexpression of miR-144 and miR-451, either separately or together, failed to restore the effects of GATA-1 loss [25]. miR-451 is also linked to other erythroid transcription factors. It was shown to directly interact with GATA-2 mRNA via its 3'UTR [26]. This shows how this miRNA can fine-tune gene expression at late stages of erythroid differentiation, as it is known that GATA-2 is gradually replaced by GATA-1 in the terminal stages of red blood cell development [27].
Peripheral blood analysis of samples from miR-144/451$^{-/-}$ mice showed mild anemia with a reduced red cell count and lower hematocrit and hemoglobin values. Mutant mice also displayed splenomegaly and anisocytosis, and suffered from ineffective erythropoiesis in their bone marrow with a decreased frequency and count of mature orthochromatophilic erythroblasts, such as Ter119$_{hi}$ and CD71$_{neg}$ [28]. Another mechanism by which miR-451 regulates erythropoiesis is through direct interaction with mouse *Ywhaz* mRNA [23, 29]. This gene encodes protein 14-3-3ζ, which takes part in the assembly of signaling complexes of pathways downstream of the growth factor receptors and modulates protein-protein interactions and subcellular localization of their targets. Its downregulation is required to protect cells against oxidative stress [30]. miR-144/451$^{-/-}$ mice treated with phenylhydrazine (PHZ), an oxidant that denatures hemoglobin, exhibited a rapid decrease in hematocrit and a delayed recovery compared to the

Table 1. Variations in miRNA expression levels depending on day of erythropoiesis and the genes with transcripts that are being regulated. The triangles represent a decrease and an increase in the expression level accordingly.

miRNA	Regulated gene transcript	Regulation of expression level depending on day of erythropoiesis	References
miR-191	*Riok3 Mxi1*	*No data*	[21]
miR-145	*TIRAP TRAF6*	▼ - / *No data*	[46, 48]
miR-144	*NRF2 Klfd*	*No data* / ▲ at least 20-fold** / *No data*	[31, 32]
miR-96	*γ-globin ORF mRNA*	*No data*	[2]
miR-376a	*CDK2 AGO2*	▼ at least 1-fold* / *No data*	[47]
miR-146a	*CXCR4 TIRAP TRAF6*	▼ at least 2-fold* / *No data*	[46, 48, 49]
miR-24	*ACVR1B (ALK4)*	▲ - ▼ - / *No data*	[22]
miR-126	*Myb PTPN9*	▲ at least 2-fold** / *No data*	[8, 46, 50]
miR-451	*Ywhaz gata2 GATA2*	▲ at least 35-fold**	[20, 23, 25, 26, 29]
miR-150	*MYB*	▼ at least 20-fold*	[20, 24]
miR-155	*ETS1 MEIS1*	▼ at least 6-fold*	[20, 51]
miR-223	*LMO2 NFI-A*	▼ - / *No data*	[36, 52]



* In comparison to the highest detected level, ** In comparison to the lowest detected level

control group. This indicates enhanced hemolysis of mutant erythrocytes and disturbed maturation of red blood cells. Exposure to H_2O_2 resulted in an accumulation of ROS and enhanced hemolysis. In addition, loss of the miR-144/451 locus resulted in reduced nuclear accumulation of Forkhead box protein O3 transcription factor (FoxO3) [23]. It has been shown that in normal erythropoiesis, from day 7 to 21, miR-451 is upregulated ~35-fold (Table 1) [20].

A recognized function of miR-144 is modulating the cell response to oxidative stress. Its direct target is the *NRF2* gene transcript. *NRF2* encodes the transcription factor that recognizes the ARE elements in the promoter regions of genes encoding the antioxidant and detoxifying enzymes. One of the processes regulated by NRF2 is the regeneration of GSH. Transfection of K562 cells with miR-144 followed by H_2O_2 treatment improves the cell response to oxidative stress [31]. Another function of miR-144 is the regulation of α-globin E1

expression by mutual interactions with the *klfd* gene transcript and promoter sequence (CACCC) in the *mir144* gene [32].

miR-221/222

Other miRNAs with known functions in erythropoiesis are miR-221 and miR-222. They are gradually downregulated from day 11 to the terminal stages of differentiation (Table 1) [20]. These miRNAs are clustered on the X chromosome and known to directly interact with the *KIT* gene transcript. A decline in the expression of these miRNAs during maturation leads to the expansion of early erythroblasts. Therefore, miR-221 and miR-222 inhibit normal erythropoiesis. These miRNAs also have potential in therapy since they block the proliferation of the kit+ TF-1 erythroleukemic cell line [33].

miR-150

A decline in miR-150 is required for erythroid differentiation. In normal mouse erythropoiesis, the expression of miR-150 progressively decreases from day 9 (Table 1) [20]. miR-150 directly interacts with *Myb* mRNA and functions as a switch for myeloid-erythroid progenitor cells (MEP), directing them towards erythrocytes or megakaryocytes. Gain and loss of function experiments showed that miR-150 drives MEP differentiation toward megakaryocytes at the expense of erythroid precursors. Experimental data also demonstrate that mice treated with PHZ show significantly decreased miR-150 expression due to increased demand for erythropoiesis [24].

miR-15a

miR-15a probably contributes to cell cycle control and functions as an anti-oncogene. Its confirmed target is *MYB*. Overexpression of miR-15a in K562 cells increases the percentage of the cells to be blocked in G_0/G_1 phase compared to the control (48 and 35% respectively) and inhibits colony formation by the hematopoietic progenitors [34].

miR-24

miR-24 inhibits the differentiation of K562 cells, erythroid colony formation and the maturation of human CD34+ hematopoietic progenitor cells by interfering with activin signaling (Table 1). Inhibition occurs via the binding of the 3'UTR of the human *ALK4* gene transcript, which represses the translation of activin type I receptor protein [22].

miR-let-7d

The involvement of miRNAs in iron transport and metabolism in erythrocytes has not been validated. miR-let-7d is the exception, as it impairs the differentiation of K562 cells by accumulation of iron in the endosomes. It has been documented that it directly interacts with the erythroid *DMT1* gene transcript, which lacks an iron regulatory element (-IRE), which encodes divalent metal ion transporter 1 (Table 1) [35].

miR-223

A decline in miR-223 expression is required for erythrocyte proliferation and differentiation at the progenitor and precursor level, resulting in a decreased fraction of mature orthochromatic erythroblasts (Table 1). Its validated target is *LMO2*, which encodes a protein that is a part of a multimeric, transcriptional, DNA-binding complex responsible for regulating the expression of genes involved in erythroid differentiation. Downregulation of miR-223 is an important step in promoting erythropoiesis, favoring the translation of one of the key functional target proteins, LMO2 (LIM-only protein 2, RBNT2) [36].

miR-191

miRNA-191 plays an important role in erythroblast enucleation. It has been shown to directly interact with mouse *Riok3* and *Mxi1* transcripts. Knockdown of *Riok3* or *Mix1* or overexpression of miR-191 blocks downregulation of *Gcn5*, thereby blocking erythroblast enucleation [21].

miR-96

Studies showed that some miRNAs are significantly more abundant in adult reticulocytes than in those obtained from umbilical cord blood. One of these is miR-96. It is also known that hemoglobin F (HbF, $\alpha_2\gamma_2$) dominates in the fetus and is replaced by hemoglobin A (HbA, $\alpha_2\beta_2$) and to a lesser extent hemoglobin A2 (HBA2, $\alpha_2\delta_2$) in adults [37, 38]. *In vitro* experiments showed that overexpression of miR-96 decreased γ-globin expression by 50%, whereas its knockdown increased γ-globin expression by 20% [2]. What distinguishes it from the previously described miRNAs is that its binding site is located within the ORF of γ-globin mRNA.

CONCLUSION

The described phenomena of RNAi prove that the role of RNA in the cell is not restricted to protein synthesis (mRNA, tRNA) or stabilization of multi-enzyme complexes (ribosomes, spliceosomes). Just like proteins, non-coding RNAs decide about the time, place and order of genes to be expressed. miRNAs are one of the most interesting parts of the RNAi mechanism to which siRNA [39], ha-siRNA [40, 41], ta-siRNA [42], nat-siRNA [43], scn-siRNA [44] and piRNA [45] belong.

The role of miRNAs in the regulation of proliferation of the hematopoietic cell line has been studied for just eight years. The role of miRNAs in erythropoiesis is rather poorly documented compared to the current state of knowledge on the lymphoid line. The significant levels of miRNA expression during the differentiation of the erythroid line prove their essential role in the process. Much current research attempting to document the activity of miRNAs in hematopoietic cell lines is based on the prediction of miRNA-mRNA interactions, for example those detailed at targetscan.org, pictar.org and microrna.org. A separate set of data comes from the global analyses of miRNAs

at various stages of differentiation [46, 47]. The dynamic growth of available data will permit an increase in the accuracy of predictions of miRNA-mRNA interactions. Because of the potential of the practical application of RNAi technology, it will shape the direction of research in the coming years.

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