MicroRNAs (miRNAs) represent a class of small non-coding regulatory RNA molecules that play important roles in normal hematopoiesis, including erythropoiesis. Although studies have identified several miRNAs that regulate erythroid commitment and differentiation, we do not understand the mechanism by which the crucial erythroid transcription factors, GATA-1 and NF-E2, directly regulate and control the commitment and differentiation, we do not understand the mechanism by which the crucial erythroid transcription factors, GATA-1 and NF-E2, directly regulate and control the erythroid differentiation of K562 cells. Furthermore, the increase of miR-199b-5p in erythroid cells occurred in a GATA-1- and NF-E2-dependent manner during erythocyte maturation. Both GATA-1 and NF-E2 bound upstream of the miR-199b gene locus and activated its transcription. Forced expression of miRNA-199b-5p in K562 cells affected erythroid cell proliferation and maturation. Moreover, we identified c-Kit as a direct target of miR-199b-5p in erythroid cells. Taken together, our results establish a functional link among the erythroid transcription factors GATA-1/NF-E2, miR-199b-5p and c-Kit, and provide new insights into the coupling of transcription and post-transcriptional regulation in erythroid differentiation.

INTRODUCTION

Hematopoiesis is the process by which all of the distinct cell lineages that form the blood and immune systems are generated from a common pluripotent stem cell type (Huang et al., 2011). Normal erythropoiesis in adult humans generates approximately 10^11 new red blood cells each day via the commitment of hematopoietic stem cells into erythroid progenitors, which subsequently differentiate into mature cells (Wang et al., 2011). Although hematopoiesis involves a variety of modulators that are influenced by each other in a complex manner, the central role of transcription factors in this bioprocess has been highlighted to date (Fukao et al., 2007).

For example, several critical transcriptional factors, such as GATA-1 and NF-E2, are indispensable for erythropoiesis, because they regulate the expression of multiple erythroid genes (Zhang et al., 2010).

MicroRNAs (miRNAs) are endogenous single-stranded non-coding RNA molecules of 19-25 nucleotides that control gene expression primarily at the post-transcriptional level by binding to the 3'untranslated region (UTR) of target mRNAs to regulate their stability and translation (Faraoni et al., 2012). For example, miRNA-96 directly suppresses gamma-globin expression and contributes to HbF regulation (Azzouzi and Wollscheid, 2011). miR-150 is up-regulated during B and T cell maturation (Navarro and Lieberman, 2010).

miR-451 is required for zebrafish and mouse erythroid development (Pase et al., 2009; Yu et al., 2010). miR-125b overexpression causes leukemia in mice (Bousqueta et al., 2010). miR-223 has been shown to influence granulocytic differentiation (Bellon et al., 2009). miR-126 and miR-150 target c-myb and result in altered megakaryocyte-erythroid cell fate (Grabher et al., 2011).

In the present study, we identified miR-199b-5p as a positive erythroid regulator, that was regulated by the key erythroid transcription factors GATA-1 and NF-E2. The up-regulation of miR-199b-5p during erythroid differentiation was dependent on the binding of GATA-1 and NF-E2 to its gene locus, which activated its transcription and maintained its high expression level in mature erythroid cells. Moreover, miR-199b-5p directly repressed c-Kit expression to promote erythroid maturation. In brief, our study successfully identified erythroid miR-199b-5p as an important regulator of human erythropoiesis.
The Neon by Dharmacon and transfected into K562 cells (100 nM) using GATA-1 or NF-E2) and control siRNA pools were synthesized for induction using hemin. siRNAs smart pools (specifically for The K562 cells were washed the next day with PBS and plated transfected into K562 cells at a final concentration of 60 nM. control molecules were obtained from Dharmacon (USA) and MiRNA-199b-5p mimic, miRNA-199b-5p inhibitor and negative Oligonucleotides and transfection media with 10% FBS. American Type Culture Collection and were cultured in DMEM detect hemoglobin-positive cells. 293T cells were obtained from hemin (Sigma-Aldrich, Germany) was added to the medium for duration of the experiment. Benzidin staining was used to hemin treatment. It was found that miR-199b-5p expression was continuously increased during K562 erythropoiesis (Fig. 1B), while other miRNAs also exhibited up-regulated expression after hemin treatment (Fig. 1B). A previous study revealed the aberrantly high expression of miR-199b-5p in polycythemia vera (PV), a myeloproliferative disorder (MPD) of the blood in which the bone marrow produces too many red blood cells. Because miR-199b-5p appeared to be up-regulated in maturing erythroid cells, we hypothesized that miR-199b-5p may function to promote erythroid maturation. Taken together, these findings suggested that miR-199b-5p might act as a positive modulator of human erythropoiesis.

RESULTS

MiR-199b-5p is up-regulated during human erythropoiesis

Previously, our study screening for potential GATA-1-activated erythroid miRNAs indicated miR-199b-5p as a candidate, as well as other potential candidate miRNAs including miR-652, -154, -193b, -375, -145 and -148 (Zhu et al., 2013) (Fig. 1A). Following this study, we performed another quantitative RT-PCR (q-RT-PCR) analysis to characterize the expression of these selected miRNAs in K562 cells undergoing erythropoiesis via hemin treatment. It was found that miR-199b-5p expression was continuously increased during K562 erythropoiesis (Fig. 1B), while other miRNAs also exhibited up-regulated expression after hemin treatment (Fig. 1B). A previous study revealed the aberrantly high expression of miR-199b-5p in polycythemia vera (PV), a myeloproliferative disorder (MPD) of the blood in which the bone marrow produces too many red blood cells. Because miR-199b-5p appeared to be up-regulated in maturing erythroid cells, we hypothesized that miR-199b-5p may function to promote erythroid maturation. Taken together, these findings suggested that miR-199b-5p might act as a positive modulator of human erythropoiesis.

Erythroid transcription factors GATA-1 and NF-E2 reside and activate miR-199b-5p expression

GATA-1 is an essential hematopoietic transcription factor that participates in the expression of numerous genes involved in erythropoiesis. GATA-1-null mice exhibited a complete ablation of erythropoiesis due to the arrested maturation and apoptosis of the erythroid precursors at the proerythroblast stage. Another transcription factor NF-E2 is also crucial for regulation of erythroid-specific gene expression. The expression of globin genes in developing erythroid cells is controlled by upstream locus control regions. The activation of these regions in vivo requires NF-E2 binding.

As erythropoiesis proceeds, the levels of GATA-1 and NF-E2 both increased (Fig. 2A), which was consistent with the expression pattern of miR-199b-5p (Fig. 1B), suggesting a association between the regulation of these two transcription factors and miRNAs. In order to examine whether GATA-1 and NF-E2
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Affect miR-199b-5p expression in erythroid cells, we treated K562 cells with either DNA constructs expressing GATA-1/NF-E2 or siRNAs specific to GATA-1/NF-E2 to achieve the overexpression or knock-down of transcription factors (Fig. 2B). The results of Western blot analysis indicated that GATA-1 was overexpressed ~3-fold in pcDNA-GATA-1 transfected K562 cells compared to the empty vector-treated cells. Inversely, the GATA-1 expression level was reduced to ~40% upon siRNAs treatment. Similarly, NF-E2 expression increased ~4-fold in overexpression assay and decreased ~2-fold in the knockdown experiment. Accordingly, miR-199b-5p increased in both GATA-1 and NF-E2 overexpressing K562 cells, whereas it was inhibited in GATA-1 and NF-E2 knock-down K562 cells (Fig. 2C). These results indicated that the expression of miR-199b-5p was controlled by GATA-1 and NF-E2.

Furthermore, in our attempt to investigate the potential binding sites of GATA-1 and NF-E2, a Transcription Element Search System (TESS, http://www.cbil.upenn.edu/cgi-bin/tess) mediated sequence analysis was performed and revealed putative GATA-1 and NF-E2 binding sites scattered within the upstream region of the human miR-199b locus (Fig. 3A). ChIP-PCR analysis was used to validate the binding and showed that both GATA-1 and NF-E2 occupied upstream of miR-199b-5p in 48 h hemin-treated K562 cells (Fig. 3B). Taken together, these results indicated that GATA-1 and NF-E2 resided and activated miR-199b-5p expression in erythroid cells.

MiR-199b-5p promotes erythroid differentiation of K562 cells
To examine whether miR-199b-5p affect erythroid differentiation, we transfected a miR-199b-5p mimic and a negative control into K562 cells. These K562 cells were then induced to undergo erythroid differentiation via hemin treatment. The influence of K562 cell erythroid differentiation was detected with benzidine (DAB) staining, fluorescence activated cell sorting (FACS) and q-PCR analysis respectively. The results of DAB staining conformed that miR-199b-5p overexpression (Fig. 4A) increased the proportion of benzidine-positive K562 cells after hemin treatment (Figs. 4B and 4C).

Moreover, gamma-globin expression was clearly up-regulated in miR-199b-5p overexpressing K562 cells compared to the negative control at 48h (Fig. 4D; Supplementary Fig. S1A). To further test and verify our finding, we analyzed the expression of two major erythroid cell surface markers (CD71/CD235a) with FACS. The overexpression of miR-199b-5p raised the percentage of CD71+/CD235a+ cells significatively compared to the negative control (Fig. 4E). In contrast, the inhibition of endogenous miR-199b-5p using miRNA inhibitors markedly repressed the erythroid differentiation of K562 cells (Fig. 4F; Supplementary Fig. S1B), as supported by decreased percentage of CD71+/CD235a+ cells (Fig. 4G), and reduced gamma-globin expression (Supplementary Figs. S1C and S1D) compared to the negative control. Taken together, these results demonstrated that miR-199b-5p promoted erythroid differentiation in K562 cells.

MiR-199b-5p represses c-Kit to regulate erythropoiesis
MiRNAs regulate specific mRNA targets by interfering their stability or repressing their translation. Therefore, we used the TargetScan and PicTar algorithms to obtain potential miRNAs containing miR-199b-5p-binding sites. mRNA targets related to erythropoiesis and hit by both algorithms were considered as candidates and were subjected to immunoblotting assays (Fig. 4E).
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Fig. 2. Identification of GATA-1/NF-E2-activated miR-199b-5p in K562 cells. (A) Western blot revealing the levels of GATA-1 and NF-E2 were both increased in hemin-treated K562 cells. The signals are normalized to GAPDH. (B) Western blot analysis of K562 cells transfected with a pcDNA construct overexpressing GATA-1/NF-E2 or siRNAs specific to either GATA-1/NF-E2 to achieve the overexpression or knock-down of these transcription factors. (C) Real-time PCR analysis of miR-199b-5p expression in K562 cells with GATA-1/ NF-E2 overexpression or knockdown.

Fig. 3. GATA-1 and NF-E2 were located on the upstream of miR-199 and activated its expression during erythropoiesis. (A) Representation of the GATA-1 and NF-E2 motifs upstream of the human miR-199 locus. (B) ChIP-PCR analysis of the GATA-1 and NF-E2 hits upstream of the miR-199 in K562 cells.
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5A; Supplementary Table S2). Besides, c-Kit was predicted to be a potential target of miR-199b-5p by TargetScan (Fig. 5A). The stem cell factor receptor (SCF) c-Kit plays a vital role in modulating cell proliferation and survival in various cell types. In particular, c-Kit is obligatory for the early amplification of erythroid progenitors. However, as the erythroid cell enters in maturation stage, c-Kit disappears from the cell surface (D’Allard et al., 2013). Therefore, although c-Kit was only predicted by the TargetScan algorithm, its role in erythroid differentiation made it an additional candidate that was examined in subsequent assays (Fig. 5A). To confirm the regulatory interactions between miR-199b-5p and its potential targets, we evaluated the effects of miR-199b-5p overexpression on the endogenous target protein levels in K562 cells. As expected, the ectopic expression of miR-199b-5p in K562 cells reduced c-Kit, TAF9B and CCNL1 expression levels by ~2-fold, ~3-fold and ~4-fold, respectively, compared to the control (Fig. 5B), whereas the expression levels of GRB10 and NLK were not changed after miR-199b-5p overexpression. Thus, our attempt to investigate the regulatory mechanism of miR-199b-5p during erythropoiesis led to the identification of its potential target genes, including c-Kit, TAF9B and CCNL1.

DISCUSSION

MicroRNAs are negative regulators of gene expression that have garnered great interest due to their role as post-transcriptional regulators of genes involved in numerous physiologic and developmental processes (Heuston et al., 2011). Differential miRNA expression is largely controlled by various transcription factors. In our previous studies, for example, miR-223 reversibly regulates the erythroid and megakaryocytic differentiation of K562 cells via down-modulation of LMO2. miR-376 down-regulates Ago2 and CDK2 which inhibit progenitor cell growth and differentiation (Wang et al., 2011). MiR-23a is a critical erythroid miRNA gene that not only is a positive regulator of erythroid differentiation but is also activated by GATA-1. In this study, we chose a potential erythroid-associated miRNA, miR-199b-5p to validate its expression, function, regulation and mechanism during erythropoiesis. MiR-199b-5p was up-regulated during K562 erythropoiesis. Two erythroid transcription factors GATA-1 and NF-E2 occupied the upstream of miR-199b locus and activated its transcription. Moreover, miR-199b-5p could promote erythroid differentiation by repressing c-Kit expression in K562 cells.
MiR-199b-5p has been discussed in various biological systems, including chronic myeloid leukemia, medulloblastoma, renal cell carcinoma and colorectal cancers (Andolfo et al., 2012; Flamant et al., 2010; Garzia et al., 2009; Wu et al., 2012). In different tissue types, miR-199b-5p acts distinct activities and is coordinated with specific transcription factors (D’Allard et al., 2012; Fang et al., 2013). One study by Bruchova et al. (2008) revealed that miR-199b expression was markedly increased in patients suffering from PV compared to healthy controls. PV is a clonal myeloproliferative disorder (MPD) caused by somatic mutations that arise in a hematopoietic multipotent cell. Hematopoiesis in PV is characterized by the accumulation of phenotypically normal erythrocytes. Thus, our finding of accumulated miR-199b-5p in matured erythroid cells was in consistent with the PV phenotype, which also suggesting miR-199b-5p as a potential therapy target in PV treatment.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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