Defective erythroid differentiation in miR-451 mutant mice mediated by 14-3-3ζ

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Erythrocyte formation occurs throughout life in response to cytokine signaling. We show that microRNA-451 (miR-451) regulates erythropoiesis in vivo. Mice lacking miR-451 display a reduction in hematocrit, an erythroid differentiation defect, and ineffective erythropoiesis in response to oxidative stress. 14-3-3ζ, an intracellular regulator of cytokine signaling that is repressed by miR-451, is upregulated in miR-451−/− erythroblasts, and inhibition of 14-3-3ζ rescues their differentiation defect. These findings reveal an essential role of 14-3-3ζ as a mediator of the proerythroid differentiation actions of miR-451, and highlight the therapeutic potential of miR-451 inhibitors.

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The formation of differentiated cell types generally requires extracellular cues that culminate in the nucleus to control the sets of genes involved in specialized cellular functions. Recently, it has become apparent that microRNAs (miRNAs) provide an important layer of control to regulatory networks for cell differentiation (Liu and Olson 2010; Zhao et al. 2010). miRNAs are noncoding RNAs (miRNAs) that influence gene expression by inhibiting translation or inducing mRNA degradation. Specific miRNAs are targeted by complementary Watson-Crick base-pairing with sequences in the 3′ untranslated region (3′ UTR) of target mRNAs (Ambros 2001).

Erythropoietin (EPO) plays a central role in the process of erythroid differentiation, during which primitive erythroid progenitors differentiate into mature enucleated erythrocytes (Constantinescu et al. 1999). EPO binding to the EPO receptor results in the activation of multiple major signaling cascades. STAT5-dependent transcription of the anti-apoptotic gene Bcl-XL, phosphatidylinositol 3 kinase (PI3K) regulation of AKT and the transcription factors GATA1 and Foxo3a, and activation of the Ras/MAPK cascade are essential intracellular mediators of EPO-induced signal transduction (Zhang and Lodish 2007). GATA1, a transcription factor essential for hematopoiesis, regulates the expression of miR-144 and miR-451, which are encoded by a bicistronic transcript expressed selectively in erythroid cells. These miRNAs have been implicated in erythroid differentiation, consistent with the central role of GATA1 in this process (Dore et al. 2008; Fu et al. 2009; Pase et al. 2009; Papapetrou et al. 2010). miR-144 has been suggested to regulate α-hemoglobin expression in zebrafish by targeting the erythroid-specific transcription factor KLF4 (Fu et al. 2009). Similarly, combined knockdown of miR-144 and miR-451 in mouse bone marrow using an unconventional approach that employs miRNA “decoys” results in abnormal erythroid differentiation, suggesting cooperative regulation of this process by both miRNAs (Papapetrou et al. 2010). Overexpression of miR-451 in the mouse erythroleukemia (MEL) cell line and the human K562 leukemic cell erythroid line results in the induction of erythroid differentiation (Bruchova-Votavova et al. 2010). Similarly, overexpression of miR-451 but not miR-144 in miR-144/145 mutant zebrafish rescues erythropoiesis (Dore et al. 2008; Pase et al. 2009). It has been proposed that miR-451 regulates erythrocyte differentiation by repressing GATA2, a pro-stem cell transcription factor (Pase et al. 2009). However, the lack of a miR-451 target sequence within the 3′ UTR of GATA2 mRNA in mammals suggests that miR-451 may function by alternate mechanisms. Overall, considerable ambiguity remains as to the role of miR-451 in mammalian erythroid differentiation, as well as the potential downstream targets that may mediate its actions.

To investigate the functions of miR-451 in vivo, we generated mice lacking miR-451. miR-451−/− mice display a mild reduction in hematocrit, accompanied by expansion of the colony-forming unit erythroid (CFU-E) progenitor cell population in bone marrow and spleen, as well as a reduced capacity to amplify erythrocyte cell number in response to oxidative stress. Transient inhibition of miR-451 in adult mice with an antagonist induces similar defects. miR-451 targets the mRNA encoding 14-3-3ζ, a chaperone protein that modulates intracellular growth factor signals at multiple steps (Silva et al. 2000; Barry et al. 2009). Thus, miR-451−/− erythroblasts express 14-3-3ζ at elevated levels, and inhibition of 14-3-3ζ rescues their erythropoiesis. Our results identify 14-3-3ζ as a key target whereby miR-451 regulates erythropoiesis, and provide the first demonstration of an antagonist mimicking the genetic deletion of a miRNA in vivo.

Results and Discussion

miR-144 and miR-451 are transcribed as a bicistronic transcript that gives rise to the two mature miRNAs (Fig. 1A;
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Dore et al. 2008). miR-451 expression overlaps with that of miR-144, with the highest expression in bone marrow and spleen [Fig. 1B]. It is apparent that miR-451 and miR-144 exhibit disparate characteristics when analyzed by Northern blot analysis. Recent studies have characterized the unique Dicer-independent biogenesis of miR-451 (Cheloufi et al. 2010; Cifuentes et al. 2010). In this regard, miR-451 migrates as a ladder upon gel electrophoresis, due to multiple 3′ termini (Cheloufi et al. 2010).

To further characterize the expression of the miR-451 locus in vivo, we generated transgenic mice in which β-galactosidase [β-gal] was expressed under the control of a conserved 5-kb promoter/enhancer region containing the previously characterized GATA-binding sites (Supplemental Fig. 1; Dore et al. 2008). Whole-mount analysis revealed β-gal reporter expression throughout the circulatory system [Fig. 1C]. Sections through the intersomitic veins indicate that this expression is due to β-gal positivity of circulating erythrocytes [Fig. 1E].

To generate miR-451 mutant mice, loxP sites for Cre-mediated recombinase were introduced at both ends of the pre-miR-451 coding region, leaving pre-miR-144 unmodified. The mutation removed 76 base pairs [bp] of genomic DNA encompassing pre-miR-451 [Supplemental Fig. 2A,B]. Deletion of miR-451 was confirmed by genomic PCR analysis [Supplemental Fig. 3].

Northern blot analysis of bone marrow harvested from 8-wk-old mice showed a 50% reduction in miR-451+/−-animals and the absence of miR-451 in miR-451−/−-animals. miR-144 levels were unchanged across all genotypes [Supplemental Fig. 2C]. Real time RT–PCR on fetal liver cells positive for the mature erythrocyte marker TER119 confirmed these results [Supplemental Fig. 2D]. miR-451−/−-animals were obtained at predicted Mendelian ratios and displayed no overt abnormalities up to 8 mo of age [Supplemental Table 1]. However, we found a mild reduction in hematoctrit of adult miR-451−/−-mice compared with miR-451+/+ littermates, suggesting an erythrocyte defect [Fig. 1D].

To examine the effect of miR-451 loss of function on erythroid differentiation, livers were isolated from embryos at 14.5 and 16.5 d post-coitus [dpc] from miR-451−/−-intercrosses. Erythrocyte development was assayed by flow cytometry analysis based on the expression of CD71 and TER119, as described previously [Socolovsky et al. 2001]. Fetal liver cells isolated from miR-451−/−-embryos showed a significant decrease in the region IV CD71/TER119 cell population, representing the most mature erythrocytes [Fig. 2A, Supplemental Fig. 3]. miR-451−/−-embryos displayed a haploinsufficient phenotype, with ~50% of wild-type levels of region IV CD71/TER119+ cells [Fig. 2B]. Haploinsufficient phenotypes are not typically observed in mice heterozygous for other miRNA-null alleles, highlighting the dependence of erythroid differentiation on miR-451 levels.

To determine whether the differentiation defect observed in miR-451−/−-mice reflected an erythroid cell-autonomous function of miR-451 versus a disruption of microenvironmental cues within the hematopoietic niche, in vitro differentiation assays were performed on
451 dpc fetal liver cells as described previously (Zhang et al. 2003). This assay replaces endogenous cues with exogenously supplied growth factors, allowing for standardization of the microenvironment. In vitro differentiation revealed a decrease in the CD71+/TER119+ cell population from cell suspensions isolated from miR-451−/− mice when compared with wild-type littermates, indicating that the differentiation defect observed in vivo is erythrocyte-autonomous (Fig. 2C,D).

To determine the effect of miR-451 ablation on erythrocyte differentiation in adult hematopoietic tissues, flow cytometry analysis was performed. miR-451−/− animals displayed a reduction in region IV CD71+/TER119+ erythrocytes and a concomitant increase in region II CD71+/TER119+ erythroblasts in bone marrow (Figs. 3A,B). This defect likely contributes to the observed reduction in the hematocrit of miR-451−/− animals compared with miR-451+/+ littermates.

Histologic analysis of spleens isolated from miR-451+/+ and miR-451−/− animals revealed an increase in cellularity in the mutants with hypercellular clusters of erythroid colonies (Fig. 3C, Supplemental Fig. 5). We measured the number of erythroid progenitors in spleen and bone marrow directly by performing colony-forming assays for burst-forming unit erythroid (BFU-E) and CFU-E progenitors ([Fig. 3D]). miR-451−/− animals displayed dramatically increased numbers of CFU-E progenitors in bone marrow and spleen; however, BFU-E progenitors were similar in both miR-451−/− and miR-451+/+ littermates. Consistent with the expansion of immature erythroid precursors observed by flow cytometry analysis, these data suggest that the erythrocyte differentiation defect results in the expansion of the early erythroblast population. This expansion may be due to the loss of miR-451, or it may reflect a response to increased EPO secretion secondary to an anemic state. Finally, the cell-autonomous differentiation defect observed embryonically and the maintenance of this phenotype throughout adulthood suggest a constitutive need for miR-451 specifically in the process of erythrocyte differentiation.

A low rate of erythropoiesis is required to maintain a steady-state hematocrit in adult mice compared with embryonic hematopoietic tissue. Therefore, the mild reduction in hematocrit of adult miR-451−/− animals likely reflects the ability of miR-451−/− hematopoietic tissues to compensate and achieve a steady-state output of erythrocytes. To examine the ability of adult hematopoietic tissues to increase their erythropoietic rate in the absence of miR-451, we subjected miR-451−/− and miR-451+/+ animals to hemolytic anemia by injecting them with phenylhydrazine hydrochloride (PHZ) (Socolowsky et al. 2001). Hematocrit and reticulocyte levels were monitored on days 0, 3, 6, and 9 thereafter. The decrease in hematocrit was greater in miR-451−/− animals compared with miR-451+/+ littermates, reflecting a hypersensitivity of mutant erythrocytes to PHZ-induced damage. miR-451−/− animals displayed a delay in recovery (Fig. 3E), indicative of a defect in generating a high erythropoietic rate. These data are consistent with results indicating that miR-451 regulates the erythrocyte response to oxidative stress, and indicate a constitutive requirement for miR-451 in the process of erythrocyte differentiation (Yu et al. 2010).

The erythrocyte differentiation defect observed in miR-451−/− animals could, in principle, result from a developmental insult or the chronic absence of miR-451. To test whether acute inhibition of miR-451 was also sufficient to impede erythroid differentiation, we performed injections of wild-type animals with a cholesterol-modified miR-451 antagomir that inhibits miRNA function by Watson-Crick base-pairing to the mature miRNA (Krutzfeldt et al. 2005). miR-451 antagomir-treated animals displayed a 90% reduction of miR-451 in bone marrow and complete loss in other tissues (Fig. 4A; Supplemental Fig. 6). Antagomir-451-injected animals also displayed an increase in region II CD71+/TER119+ erythroblasts, with a concomitant decrease in region IV CD71+/TER119+ erythrocytes, suggesting that acute inhibition of miR-451 function results in the rapid block of erythrocyte differentiation, as observed in the miR-451−/− animals (Fig. 4B). These data suggest that miR-451 plays a constitutive role in the differentiation of erythrocytes, and that miR-451−/− erythrocytes display this defect due to the chronic requirement for miR-451 rather than an embryonic insult. These findings also demonstrate that chronic versus transient loss of miRNA function has similar consequences. This is the first study concomitantly describing the same phenotype in both miRNA genetic-null animals and animals injected with miRNA inhibitors.

To determine the mechanism of miR-451-mediated erythroid differentiation, we compared gene expression profiles of CD71+/TER119+ erythroblasts from miR-451−/−
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Figure 4. miR-451 inhibition with antagomir-451 rapidly induces a defect in erythroid differentiation. [A] Northern blot analysis of miR-451 in bone marrow of animals injected with either saline, mismatch, or antagomir-451 shows 90% knockdown of miR-451. [B] FACS analysis was performed on bone marrow stained with CD71 and TER119. Percentage of cells gated are displayed within the respective gate

miR-451+/− fetal liver cells at 14.5 dpc. miR-451 expression is absent in TER119+/− erythroblasts [Dore et al. 2008]. Therefore, the CD71+/TER119− [region II] population was chosen for analysis due to its high expression of miR-451 and its relative uniformity across genotypes at 14.5 dpc.

By microarray analysis, we found a total of 130 probe sets that were up-regulated twofold or more and 26 probe sets that were up-regulated threefold or more in miR-451−/− cells compared with miR-451+/+ cells [Supplemental Table 2]. From this list, 13 genes are predicted to be targeted by miR-451 by the target prediction algorithm microCosm [http://www.ebi.ac.uk/enright], whereas only three of these genes are predicted miR-451 targets by the Targetscan algorithm [http://www.targetscan.org], which more stringently considers cross-species conservation. These three genes (ywhaz, cab39, and vapa) contain miR-451-binding sites conserved in mammals. Of these genes, ywhaz, which encodes the chaperone protein 14-3-3, was up-regulated most dramatically. Due to the high conservation of the 3′ UTR, the magnitude of up-regulation of ywhaz, and the established role of 14-3-3 as a modulator of hematopoiesis, we hypothesized that ywhaz was primarily responsible for the defect observed in miR-451−/− animals [Stomski et al. 1999; Sliva et al. 2000; Barry et al. 2009]. The miR-451 target sequence in ywhaz 3′ UTRs is conserved across species [Supplemental Figure 7A]. Gene ontology analysis using the database for annotation, visualization, and integrated discovery (DAVID) [Dennis et al. 2003] algorithm revealed perturbations of a variety of cellular processes in miR-451−/− erythroblasts [Supplemental Table 3]. Further analysis of the microarray revealed that mRNA levels of the previously published miR-451 target GATA2 were unchanged across genotypes [Supplemental Table 2].

To directly test for possible regulation of ywhaz by miR-451, TER119−/−-enriched 14.5 dpc fetal liver cells were harvested from miR-451−/− and miR-451+/+ animals, and quantitative real-time RT-PCR for six transcripts encoding 14-3-3 isoforms was performed. Of these transcripts, only ywhaz was up-regulated in miR-451−/− cells compared with miR-451+/+ cells [Supplemental Fig. 7B]. Immunoblot analysis revealed ~1.5-fold up-regulation of 14-3-3 protein levels in miR-451−/− cells and twofold up-regulation in miR-451−/− cells [Fig. 5A,B]. A luciferase reporter fused to the ywhaz 3′ UTR was repressed by miR-451 in COS-1 cells, and mutation of the miR-451-binding site within this UTR abolished repression by miR-451 [Fig. 5C].

14-3-3 chaperone proteins play important roles in the assembly of signaling complexes required for the coordinate activation of pathways downstream from growth factor receptors. These proteins bind phosphoserine/threonine-containing sequences, and thereby modulate protein–protein interactions and subcellular localization of their targets [Aitken 2006]. Numerous studies have implicated 14-3-3 in signal transduction and transcriptional events involved in hematopoiesis [Stomski et al. 1999; Barry et al. 2009]. 14-3-3z coordinates signal transduction downstream from hematopoietic growth factor receptors by interacting with the common signaling subunit of the granulocyte–macrophage colony-stimulating factor [GM-CSF], interleukin 3 [IL-3], and IL-5 receptors [Stomski et al. 1999], and interacts directly with the IL-9 receptor [Sliva et al. 2000]. 14-3-3z also governs P13K signaling downstream from GM-CSF, a key regulator of erythropoiesis [Barry et al. 2009], and association of 14-3-3 with Gab2, a docking protein required for hematopoiesis, terminates Gab2 function [Brunner et al. 2008].

Due to the role of 14-3-3z as a regulator of hematopoiesis, we hypothesized that repression of 14-3-3z expression...
in miR-451$$^{-/-}$$ erythrocytes would relieve the erythroid differentiation defect. To test this hypothesis, we infected 14.5-dpc TER119$$^+$$ progenitor-rich fetal liver cells with shRNAs targeting two separate regions of the ywhaz transcript, abbreviated sh-1 and sh-2, using retroviral vectors. A retrovirus expressing a shRNA directed against luciferase [sh-Luc] was used as a control. Due to the reproducible differentiation defect observed ex vivo, we used our in vitro differentiation system to assay for functional rescue of the erythrocytoid differentiation defect observed in miR-451$$^{-/-}$$ animals. Expression of GFP from an internal ribosomal entry site (IRES) allowed for the identification of viable infected cells throughout differentiation. Each shRNA directed against 14-3-3$$\zeta$$ efficiently reduced its expression [Supplemental Fig. 8]. Pooled fetal liver cells from 14.5-dpc embryos isolated from miR-451$$^{-/-}$$ intercrosses were first depleted of TER119$$^+$$ cells by MACS, separated into individual wells, and then infected with retrovirus expressing either sh-1, sh-2, or sh-Luc. Cells were then resuspended in medium containing EPO and induced to differentiate as described previously [Zhang et al. 2003]. Knockdown of ywhaz was confirmed from total cell suspensions (GFP-positive and GFP-negative cells) isolated at the time of flow cytometry analysis [Fig. 5D,E]. This shift was observed in both shRNA-treated groups and not in the sh-Luc-infected group, indicating an increase in erythrocytoid differentiation due to the specific repression of 14-3-3$$\zeta$$. These data demonstrate that repression of 14-3-3$$\zeta$$ enhances erythrocytoid differentiation in the absence of miR-451, and strongly supports the conclusion that miR-451 repression of 14-3-3$$\zeta$$ enhances erythrocytoid differentiation.

Loss of function and gain of function of specific effectors downstream from the EPO receptor typically result in erythrocyte defects [Zhang and Lodish 2007]. Loss of K-ras diminishes AKT activation, and thereby results in a mild erythrocytoid differentiation defect [Zhang and Lodish 2005]. Conversely, overexpression of factors that exaggerate intracellular signaling similarly induce erythrocytoid differentiation defects. Chronic activation of STAT5, AKT, and p44/42 MAPK in erythroid progenitors by K-ras expression leads to a mild block in terminal erythrocytoid differentiation, and expansion of erythroid progenitors [Zhang et al. 2007]. Hyperactivation of these pathways blocks terminal erythrocytoid differentiation and leads to cytokine-independent growth of erythroid progenitors [Braun et al. 2004]. Due to the essential function of 14-3-3$$\zeta$$ as an effector of PI3K/AKT pathways, it is likely that its misregulation in erythroid progenitors affects the differentiation process. Finally, our finding that inhibition of 14-3-3$$\zeta$$ in miR-451$$^{-/-}$$ animals restores erythrocytoid differentiation suggests that 14-3-3$$\zeta$$ is a key downstream effector of the proerythroblast actions of this miRNA.

Our results indicating the sensitization of miR-451$$^{-/-}$$ erythrocytes to oxidative stress are consistent with work indicating a similar phenotype in miR-144$$^{-/-}$$/miR-451$$^{-/-}$$ animals, and suggest that miR-451 rather than miR-144 regulates this process [Yu et al. 2010]. Yu et al. (2010), however, did not observe an erythrocyte differentiation defect in adult hematopoietic tissues. It is possible that this discrepancy is due to the combined knockout of both miR-144 and miR-451. miR-144 has been shown to influence hemoglobin expression; therefore, loss of miR-144 and its effects on hemoglobin may result in the activation of compensatory pathways, blunting the differentiation defect [Fu et al. 2009]. To address this possibility, a miR-144 loss-of-function model should be analyzed.

Our findings that red blood cell production shows a stoichiometric dependency on miR-451 levels, and that acute inhibition of miR-451 with an antagomir induces an erythroid differentiation defect, suggest that miR-451 oligonucleotide inhibitors may be efficacious in the treatment of red cell dyscrasias, such as polycythemia vera, or other hematopoietic malignancies. Finally, miR-451 has been reported to participate in other disorders, including glioma tumorigenesis and pulmonary hypertension [Caruso et al. 2010; Godlewski et al. 2010]. Thus, it will be of interest to determine whether inhibitor miR-451 oligonucleotides demonstrate therapeutic efficacy in these disorders.

Materials and methods

Generation of miR-451 mutant mice

pre-miR-451 was targeted by homologous recombination in embryonic stem cells. Chimeric mice were bred to C57BL/6 mice. A detailed description of miR-451 mutant generation is provided in the Supplemental Material.

Hematocrit and reticulocyte measurements

Hematocrit was measured using a hematocrit centrifuge. Reticulocytes were counted using the Miller Disc method. Details are provided in the Supplemental Material.

FACS, cell purification, and in vitro differentiation assays

Cells were analyzed by FACS analysis as described in the Supplemental Material. Cell purification was performed by the Flow Cytometry Core Facility [University of Texas Southwestern]. MACS was performed through MACS separation columns (Miltenyi Biotec). Differentiation assays were performed as described previously [Zhang et al. 2003]. Details are provided in the Supplemental Material.

Antagomir injections

Antagomir or scrambled control (80 mg/kg) was injected on days 0 and 1, and mice were analyzed on day 3.

Microarray analysis

RNA harvested from TER119$$^+$$CD71$$^+$$ cells was analyzed using Mouse Genome 430 A2.0 chips (Affymetrix). Details of analyses are provided in the Supplemental Material. Experimental procedures are described in detail in the Supplemental Material.

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