miR-144/451 represses the LKB1/AMPK/mTOR pathway to promote red cell precursor survival during recovery from acute anemia

Xiao Fang,1,2* Feiyang Shen,2* Christophe Lechaufe,3 Peng Xu,2 Guowei Zhao,3 Jacobi Itkow,2 Fan Wu,2 Yaying Hou,2 Xiaohui Wu,4 Lingling Yu,4 Huiqing Xiu,2 Mengli Wang,2 Ruiling Zhang,2 Fangfang Wang,2 Yanqing Zhang,2 Daxin Wang,1 Mitchell J. Weiss3 and Duonan Yu2,4,6,7

1Clinical Medical College of Yangzhou University, Yangzhou, China; 2Jiangsu Key Laboratory of Experimental & Translational Non-Coding RNA Research, University School of Medicine, China; 3Department of Hematology, St. Jude Children’s Research Hospital, Memphis, TN, USA; 4Department of Pediatrics, Jingjiang People’s Hospital, Yangzhou University, Jingjiang, China; 5Institute of Comparative Medicine, Yangzhou University, China; 6Institute of Translational Medicine, Yangzhou University School of Medicine, Yangzhou, China and 7Jiangsu Co-Innovation Center for Prevention and Control of Important Animal Infectious Disease and Zoonosis, Yangzhou, China

XF and FS contributed equally to this work.

ABSTRACT

The microRNAs miR-144 and -451 are encoded by a bicistronic gene that is strongly induced during red blood cell formation (erythropoiesis). Ablation of the miR-144/451 gene in mice causes mild anemia under baseline conditions. Here we show that miR-144/451 represses erythroblasts exhibiting increased apoptosis during recovery from acute anemia. Mechanistically, miR-144/451 depletion increases the expression of the miR-451 target mRNA Cab39, which encodes a co-factor for the serine-threonine kinase LKB1. During erythropoietic stress, miR-144/451 represses erythroblasts exhibiting abnormally increased Cab39 protein, which activates LKB1 and its downstream AMPK/mTOR effector pathway. Suppression of this pathway via drugs or shRNAs enhances survival of the mutant erythroblasts. Thus, miR-144/451 facilitates recovery from acute anemia by repressing Cab39/AMPK/mTOR. Our findings suggest that miR-144/451 is a key protector of erythroblasts during pathological states associated with dramatically increased erythropoietic demand, including acute blood loss and hemolytic anemia.

Introduction

Dysregulation of microRNAs (miRNAs) is implicated in the pathophysiology of many human diseases including cancer, cardiovascular disease, and anemia.1,2 There is increasing evidence that miRNAs regulate red blood cell (RBC) formation (erythropoiesis) by controlling the proliferation and differentiation of RBC precursors, termed erythroblasts.3 For example, miR-126 negatively regulates erythropoiesis by repressing mRNA encoding the tyrosine phosphatase PTPN9, which is required for erythroblast proliferation,1 whereas ectopic expression of miR-27a, miR-24, and miR-146b in CD34+ hematopoietic progenitor cells promotes erythroid maturation by repressing GATA-2 or increasing GATA-1, which activates the GATA switch, a key step in erythropoiesis.1,3,4 However, the role of miRNAs and their targets in regulating erythropoiesis is not fully understood.

The bicistronic miRNA locus encoding miR-144 and -451 is strongly induced during erythropoiesis in zebrafish, mice, and humans.5,6 Chromatin immunoprecipitation (ChIP) and gene complementation studies show that miR-144/451 transcription is activated by GATA-1,6 a transcription factor that regulates many aspects of erythropoiesis, including precursor proliferation, maturation, and survival. Remarkably, miR-451 accounts for approximately 50% of the total miRNA pool in mouse fetal liver (FL) erythroblasts.7 Unlike most miRNAs, miR-451 biogenesis occurs independently of the RNA III enzyme Dicer. Rather, it is Argonaute 2 (Ago2)
that catalyzes the cleavage of pre-miR-451 hairpins.\textsuperscript{12} Inhibition of miR-144/451 blocks erythropoiesis in tissue culture models.\textsuperscript{10,13-15} Fewer studies have been performed with in vivo models.\textsuperscript{13-15} Moreover, the phenotypes observed after manipulating miR-144/451 expression vary according to the model used and the mode of gene manipulation. For example, miR-144/451 inhibition appears to exert a greater effect on erythroblasts in culture than on those in vivo, suggesting that the phenotype depends on the cell environment (X Fang et al., unpublished data, 2017). Along with others, we have also demonstrated that miR-144/451 gene knockout (KO) mice exhibit mild baseline anemia that worsens upon oxidative stress.\textsuperscript{15,15} Similarly, loss of miR-451 in zebrafish renders erythroid precursors sensitive to oxidative stress.\textsuperscript{12} The anti-oxidant role of miR-144/451 during erythropoiesis is at least partially dependent on suppression of the mRNA target Ywhaz, which encodes the cytoplasmic adaptor protein 14-3-3ζ.\textsuperscript{13,15} miR-144/451 depletion increases 14-3-3ζ protein, which sequesters the transcription factor FoxO3 in the cytoplasm, thereby reducing expression of several target genes that encode anti-oxidant proteins. This mechanism explains the hypersensitivity of miR-144/451−/− RBCs to oxidative stress but is unlikely to account for all the activities of these miRs. For example, miR-144/451−/− mice exhibit ineffective erythropoiesis at baseline and delayed recovery after anemia caused by oxidative stress\textsuperscript{13-15} via unknown mechanisms.

We discovered that erythroblasts isolated from miR-144/451−/− FL during embryonic gestation or bone marrow and spleen during acute anemia exhibit increased apoptosis compared to wild-type (WT) counterparts. This effect is mediated by derepression of the direct miR-451 target mRNA Cab39 followed by activation of the downstream LKB1/AMPK/mTOR pathway. Thus, miR-144/451 enhances physiological responses to acute anemia by promoting the survival of RBC precursors.

Methods

Animals

miR-144/451 KO mice were described previously.\textsuperscript{15} p53ER knock-in (KI) mice were kindly provided by Gerard Evan (University of Cambridge, UK).\textsuperscript{35} KO mice were described previously.\textsuperscript{15} The database for G1E-ER4 erythroid markers 7AAD, propidium iodide (PI) or Live/Dead® Near-IR Fixable Dead Cell Stain (Invitrogen). Erythroid subpopulations were sorted on the basis of CD71/Ter119 expression (BD Biosciences). The Annexin V Early Apoptosis Detection Kit (cat. n. 555736) was obtained from BD Biosciences. The nucleation of erythroblasts was quantitated by staining with Hoechst 33342 (Sigma); cell viability was quantitated by staining cells with death markers 7AAD, propidium iodide (PI) or Live/Dead® Near-IR Fixable Dead Cell Stain (Invitrogen). Erythroid subpopulations were sorted on the basis of CD71/Ter119 expression (BD Biosciences).

Results

Increased apoptosis of miR-144/451−/− erythroblasts during erythropoietic stress

To study the effects of miR-144/451 on erythropoiesis, we cultured equal numbers of FL erythroid precursors from embryonic day (E) 14.5 KO or WT embryos in media that facilitated their expansion or terminal maturation\textsuperscript{18} (Figure 1A). After 48 hours (h) in expansion medium, miR-144/451−/− erythroblasts exhibited reduced cell num-
bers (Figure 1B) and increased apoptosis, which was quantified by Annexin V staining (Figure 1C and D). As expected, KO erythroblasts lacked miR-451 expression (Figure 1E). Both WT and KO cells maintained their erythroblast identity after 48 h in culture (Figure 1F). Similarly to the effects observed in expansion medium, miR-144/451−/− erythroid progenitors exhibited reduced cell numbers and increased apoptosis after 48 h culture in maturation medium (Figure 1G and H). Importantly, erythroid precursors isolated directly from miR-144/451−/− E14.5 FLs were

**Figure 1. Increased apoptosis of miR-144/451−/− erythroblasts during erythropoietic stress.** (A) miR-144/451−/− progenitor cells were isolated from E14.5 fetal liver (FL) erythroblasts and grown in culture in expansion medium or maturation medium. (B) Cell proliferation rates of FL erythroblasts in expansion medium for 24 and 48 hours (h), n=3 FLs. **P<0.01 (t-test). Experiments were repeated 3 times. (C) Flow cytometry-based analysis of apoptosis using Annexin V. (D) Quantitative analysis of the flow cytometry data from (C). Data from 3 independent experiments. **P<0.01 (t-test). (E) Quantitative PCR analysis of miR-451 expression in FL progenitors grown in expansion medium for different lengths of time. Progenitor cells from miR-144/451−/− FLs, mouse B-lymphoma cell line 38B9, and normal B lymphocytes sorted from mouse bone marrow were used as negative controls. Note: the high miR-451 expression in the 0-h culture suggests the erythroid identity of the progenitors, whereas the lack of a significant increase in miR-451 after 48 h in culture suggests that there is no further differentiation of erythroid progenitors grown in culture. (F) FL progenitors grown in expansion medium for 48 h. Cells were cytospun onto slides and stained with May-Grunwald-Giemsa. (G) Cell proliferation rates of FL erythroblasts in maturation medium for 24 and 48 h. N=3. **P<0.01 (t-test). (H) Percentage of apoptotic cells in maturation medium based on flow cytometric analysis. Data represent 3 independent experiments. **P<0.01 (t-test). (I) Cell numbers in whole E14.5 FLs without in vitro expansion. WT n=10, KO n=11. *P<0.05 (t-test). (J) Percentage of apoptotic cells in different regions gated by CD71/Ter119 staining and FSC intensity. WT n=7, KO n=6. *P<0.05 (t-test).
reduced in number and exhibited increased apoptosis compared to controls (Figure 1I and J). However, we detected no increase in apoptosis of erythroblasts isolated directly (not cultured) from spleen or bone marrow from adult miR-144/451−/− mice (Online Supplementary Figure S1A and B). Fetal liver erythropoiesis is considered a “stress state” because production demands are extremely high compared to steady state bone marrow erythropoiesis in adults.20 Thus, miR-144/451 may protect erythroblasts from apoptosis during erythropoietic stress associated with increased demands for RBC production. Consistent with this, we had previously noted that recovery from

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**Figure 2. Apoptotic erythroblasts in miR-144/451−/− bone marrow increase after administration of 5-fluorouracil (5-FU) in adult mice.** (A) Survival rates after 5-FU treatment for n=38 (wild-type, WT) and n=36 (miR-144/451−/−, KO) mice. (B) Increased hemolysis of miR-144/451−/− erythrocytes after exposure to 5-FU, as determined by serial hematocrit measurement. N=12 miR-144/451−/− and n=12 WT mice were used. **P<0.05; ***P<0.01 (t-test). (C) Flow cytometric analysis of Ter119+CD71+ reticulocytes in circulating blood after 5-FU treatment. N=5 mice of each genotype were used. ***P<0.001 (t-test). Note: for the first eight days there were relatively more reticulocytes in miR-144/451−/− blood as compared to WT blood, but significantly fewer during days 8 to 12. The number of reticulocytes in miR-144/451−/− blood dramatically increased around day 14, and much higher levels were sustained than in WT animals. (D) Flow cytometric analysis of Ter119+CD71+ erythroid cells in bone marrow after 5-FU administration for 7-11 days. Note: the appearance of Ter119+CD71+ erythroid cells in bone marrow was delayed relative to that in WT mice, indicating a maturation arrest and/or sustained apoptosis of erythroid cells in miR-144/451−/− mice. (E) Quantitated analysis of Ter119+CD71+ erythroid cells in bone marrow after 5-FU administration for 7-11 days. There were n=5 mice of each genotype at each time point. **P<0.01 (t-test). (F) Flow cytometric analysis of nucleated cell numbers in bone marrow after 5-FU administration. All cells shown in the windows were Ter119+. Gated regions represent nucleated erythroblasts (Hoechst+FSChigh). (G) Quantitative analysis of flow cytometry data from (F). There were n=5 mice of each genotype at each time point. ***P<0.001 (t-test). Note: there were far fewer nucleated erythroblasts in miR-144/451−/− bone marrow during days 9 to 11, indicating a maturation arrest and/or sustained apoptosis. (H) Flow cytometric analysis of early apoptosis using Annexin V. Ter119+/Hoechst+ cells are nucleated erythroid cells (left). Near-IR cell death marker/Annexin V+ cells are early apoptotic cells (right). (I) Quantitative analysis of flow cytometry data from (H). We used n=5 mice of each genotype at day 11 after 5-FU treatment. **P<0.01 (t-test).
(PHZ)-induced hemolytic anemia was delayed in miR-144/451−/− mice. To examine further the effects of erythropoietic stress on adult miR-144/451−/− mice, we treated them with a single dose of 5-fluorouracil (5-FU), which destroys committed hematopoietic progenitors, including erythroblasts. Compared to WT mice, miR-144/451−/− mice exhibited increased mortality after 5-FU treatment (55.6% vs. 86.8% survival) (Figure 2A), which was associated with a greater decline in their hematocrit (Figure 2B). During the recovery phase after 5-FU treatment, the mutant mice exhibited higher levels of late stage circulating erythroid precursors (reticulocytes) (Figure 2C), probably in response to the more severe anemia (Figure 2B). However, the time to maximal reticulocyte response was delayed by several days in KO mice compared to WT controls (Figure 2C). Similarly, the emergence of bone marrow erythroid precursors was delayed in KO mice (Figure 2D-G). We also observed increased apoptosis of miR-144/451−/− bone marrow and splenic erythroblasts during recovery from PHZ-induced anemia (Figure 2A and B) or phlebotomy (Figure 2C and D). Thus, miR-144/451−/− erythroblasts exhibit increased apoptosis under conditions of increased physiological demand for RBC production, i.e. under erythropoietic stress.

miR-451 targets Cab39 mRNA in erythroblasts

Several mRNAs previously identified as miR-451 target mRNAs, including Myc, Ywhaz/14-3-3ζ, and Cab39 (Figure 4A), encode general regulators of cell survival, proliferation, and maturation. Cab39 is an obligatory cofactor for the serine/threonine kinase LKB1, a tumor suppressor that regulates responses to metabolic stress, in part by activating AMP-activated protein kinase (AMPK). miR-451 drives human glioma cell expansion by inhibiting this pathway via direct repression of Cab39. Therefore, we investigated whether miR-451 repression of Cab39 regulates erythroblast survival during erythropoietic stress. Compared to controls, Cab39 mRNA and protein were up-regulated in miR-144/451−/− erythroblasts in spleen, bone marrow (Figure 4B and C) and FL (Figure 4D) compared to WT erythroblasts in the same tissues. Retroviral vector-mediated expression of miR-451 in the erythroid cell line G1E reduced Cab39 protein by approximately 50% (Figure 4E). The seed sequence of miR-451 is complementary to a conserved sequence within the 3′ untranslated region (UTR) of human and mouse Cab39 mRNA (Figure 4A). To verify whether miR-451 inhibited Cab39...
mRNA expression via direct interaction with this region, we fused the 3′-UTR of Cab39 mRNA to the coding sequence of luciferase cDNA (Figure 4F). In 293T cells, luciferase reporter activity was inhibited approximately 200-fold after co-expression of miR-451. Mutations in the Cab39 mRNA 3′ UTR that disrupt complementarity to the miR-451 seed sequence abrogated repression of reporter activity. Together, these findings verify that miR-451 inhibits Cab39 mRNA expression directly and that this interaction occurs during erythropoiesis.

**Activation of the Cab39/AMPK/mTOR pathway in miR-144/451−/− erythroblasts**

Cab39 binds LKB1 and STRAD to activate AMPK by phosphorylating the protein at Thr172 (Figure 5A).26,27 Of note, LKB1 and AMPK mRNAs are up-regulated during normal mouse and human erythroid maturation (Online Supplementary Figure S2A-E).19,24 Cab39 was up-regulated in cultured miR-144/451−/− FL erythroblasts compared to controls (Figures 4D and 5B and C). These cells also exhibited strongly increased phosphorylation of AMPK at Thr172, and to a lesser extent, upregulation of total AMPK protein (Figure 5B and D). AMPK can inhibit the mTOR pathway to inhibit cell growth and either induce or suppress apoptosis, depending on cellular context.25 To investigate this mechanism in miR-144/451−/− erythroblasts, we performed Western blot studies to interrogate upstream and downstream effectors.26,27 miR-144/451−/− FL erythroblasts grown in culture exhibited elevated phospho-Raptor and phospho-TSC2, along with reduced phosphorylation of p70S6K, S6, and eIF4B (Figure 5B and E-I), consistent with suppression of the mTOR by activated AMPK (Figure 5A). We observed similar patterns in primary erythroblasts from E14.5 FLs (Online Supplementary Figure S3). Together, these findings indicate that the loss of miR-144/451 during FL erythropoiesis derepresses the miR-451 target Cab39, resulting in mTOR repression.

**Attenuation of Cab39/AMPK/mTOR signaling rescues erythroid apoptosis after miR-144/451 depletion**

To determine whether miR-144/451 regulates survival of FL erythroblasts via Cab39/AMPK/mTOR, we designed shRNA-expressing retroviruses to knock down components of this pathway. We grew FL erythroblasts in expansion medium, infected them with individual shRNA retroviruses, induced erythroid maturation for 24 h, and then examined apoptosis in the cells. Inhibiting Cab39 expression in miR-144/451−/− erythroblasts by approximately 55-70% with 2 different shRNAs reduced apoptosis by approximately 45% (Figure 6A-C). In contrast, no signifi-
cant change in apoptosis of WT erythroblasts occurred after shRNA suppression of Cab39 (Figure 6B and C). Similarly, shRNA inhibition of AMPKα and TSC2 inhibited apoptosis of miR-144/451−/− erythroblasts but not WT controls (Figure 6D-G).

To further examine the effects of AMPK/mTOR signaling during WT and miR-144/451−/− FL erythropoiesis, we used drugs to manipulate the pathway. Consistent with the results of shRNA studies, the AMPK inhibitor Compound C (CC) reduced apoptosis significantly in miR-144/451−/−, but not WT erythroblasts (Online Supplementary Figure S4A and B). Conversely, inhibiting mTOR activity with rapamycin or activating AMPK with AICAR induced apoptosis in WT erythroblasts (Online Supplementary Figure S4C-F). Overall, our results with shRNAs and pharmacological inhibitors indicate that miR-451 facilitates fetal erythroblast survival by inhibiting expression of Cab39, resulting in suppression of LKB1 and AMPK and activation of the downstream mTOR pathway.

The increased apoptosis of miR-144/451−/− erythroblasts is p53-dependent

Depending on cell context, mTOR can regulate p53 positively or negatively to alter rates of apoptosis.28-30 Thus, we investigated p53 levels and effector functions in miR-144/451−/− erythroblasts that exhibit reduced mTOR activity. p53 level was increased in miR-144/451−/− E14.5 FL erythroblasts (Figure 7A). To investigate the functional implications of this finding, we crossed miR-144/451−/− mice with "p53 knock-in (KI)" mice, in which both alleles of the normal p53 gene are replaced by a cDNA encoding a 4-hydroxytamoxifen (4-OHT)-dependent form of the fusion protein, p53-estrogen receptor.16,31 There is a lack of endogenous p53 activity in the p53 KI mice unless 4-OHT is applied. In the absence of 4-OHT, loss of p53 function rescued the deficient erythroblast numbers in E14.5 FL from miR-144/451−/− mice (Figure 7B and C). Moreover, apoptosis of E14.5 miR-144/451−/− FL erythroblasts was significantly reduced in the absence of p53 activity (Figure 7D-G).
Discussion

Although the biological functions of miR-144/451 have been studied extensively, few studies have been performed in animal models. Moreover, less is known about the miR-144/451-regulated molecular pathways underlying the phenotypes observed after miR-144/451 depletion. Our results provide further explanation, in addition to that of elevated oxidative stress caused by aberrant 14-3-3ζ accumulation and consequent FoxO3 sequestration, for the defective erythropoiesis and hemolytic anemia seen in miR-144/451−/− mice.13,15 Upon various erythropoietic stresses, miR-144/451 depletion up-regulated expression of the miR-451 target Cab39 with consequent activation of the AMPK/mTOR pathway, leading to increased erythroid apoptosis. Manipulating both AMPK and mTOR activities altered the apoptotic rate in miR-144/451−/− erythroblasts. In contrast, reduced miR-451 levels in glioma cells as an adaptation to metabolic stress derepress Cab39 to activate the LKB1/AMPK/mTOR pathway and enhance survival.21 Thus, miR-451 may employ a common pathway to regulate stress responses in different cell types, but the net effects are context-dependent. During erythropoiesis, mTOR activity is relatively high, and the pathway appears to exert a positive effect on precursor expansion and protection against erythropoietic stress,22 consistent with the current study.

Contrasting effects of AMPK activity on apoptosis have been observed during various cellular stresses.25 In some cases, AMPK functions to balance cellular redox state and promote survival during metabolic or genotoxic stress.33,34 In other cases, AMPK activation during stress causes increased apoptosis.35,36 In this study, we showed that in...
miR-144/451−/− erythroblasts, AMPK is activated by overexpressed Cab39 and promotes apoptosis by inhibiting mTOR activity. It is unclear how levels of stress affect the regulatory role of AMPK in apoptosis, i.e. does AMPK favor cell survival under mild stress but enhance apoptosis under severe stress?

In this study, we found that loss of miR-144/451 induces apoptosis in a p53-dependent fashion. Some evidence from a study in Diamond-Blackfan anemia (DBA) has shown that haploinsufficiency of ribosomal protein subunits can induce erythrob last apoptosis via p53-dependent mechanisms.7 Stabilization of p53 by the MDM2 antagonist SAR405838 induces major hematopoietic defects including erythroid precursor apoptosis in vitro.28 How p53 is activated after miR-144/451 depletion remains to be elucidated. In some cell types, AMPK induces apoptosis by activating p53 through phosphorylation,34,35 whereas others have reported that p53 activates AMPK/mTOR signaling to suppress cell growth by targeting sestrin 1 and sestrin 2 upon stress.39 In addition, several groups report an inhibitory role of mTOR activity on p53 function.28, 29 It is possible that a positive feedback loop involving AMPK, p53, and mTOR signaling regulates apoptosis of miR-144/451−/− erythroid cells under stress conditions, although further investigations are required to fully define the process.

Anemia is a common complication after orthotopic kidney transplantation due to multifactorial effects including iron deficiency, reduced erythropoietin production, and chronic or acute inflammation. Additionally, mTOR inhibition by sirolimus is a possible risk factor for the development of anemia in kidney transplant recipients.40 There is also a substantial risk of anemia from the mTOR inhibitor everolimus in cancer therapy.41 Interestingly, studies in animal models and cell cultures demonstrate that treatment with mTOR inhibitors reduces RBC size, independent of alterations in kidney function.42 43 Consistent with this finding, miR-144/451−/− mice exhibit microcytic anemia,45 perhaps due to mTOR inhibition. Moreover, the current study shows that these mice exhibit enhanced erythrob last apoptosis with various erythropoietic stresses including developmental expansion of FL, hemolysis, acute blood loss, and precursor depletion by 5-FU, a chemotherapeutic drug. Accelerated apoptosis in the absence of miR-144/451 is caused by overexpression of the miR-451 target Cab39, which activates AMPK, thus inhibiting mTOR signaling. Therefore, our data explain why erythroid apoptosis is an underlying mechanism of profound anemia when the enzymatic activity of Cab39/LKB1/AMPK is significantly increased and mTOR signaling is perturbed.

A mechanistic understanding of the differences between steady state and stress erythropoiesis could be of benefit in multiple clinical settings.44 Moreover, these processes are likely to be impacted differently by various disease states. Common causes of acquired anemia include dietary iron deficiency, malaria, chronic infectious diseases, autoimmune or rheumatological disorders, chemotherapy, and chronic kidney disease. Genetic causes of anemia include DBA and hemoglobinopathies such as sickle cell disease (SCD) and thalassemia.45 Of note,

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**Figure 7.** The increased apoptosis of miR-144/451−/− erythroblasts is p53-dependent. (A) Western blot showing the protein levels of the tumor suppressor p53 in wild-type (WT) and miR-144/451−/− (KO) E14.5 fetal liver (FL) cells. Lineage negative-selected WT and KO E14.5 FL cells were grown in culture in erythroid maturation medium, and cells were harvested for Western blot analysis for p53 after 24 hours in culture. Actin was used as the loading control. (B) Gross view of the cell pellets from miR-144/451−/− and miR-144/451−/−/p53+/- mice. (C) Total erythroid cell number for whole E14.5 FLs from miR-144/451−/− and miR-144/451−/−/p53+/- FLs. Total erythropoietin (Epo) and p53 Ki double-mutant E14.5 FLs were grown in culture in erythroid maturation medium, and cells were harvested for Western blot analysis for p53 after 24 hours in culture. Actin was used as the loading control. (D) Flow cytometric analysis of apoptosis for miR-144/451−/− and miR-144/451−/−/p53+/- FLs. Total erythropoietin (Epo) and p53 Ki double-mutant mice were used. **P<0.01 (t-test). (E) Quantitative analysis of flow cytometric data from (D).
while numerous studies, including the current one, reveal a positive effect of mTOR on erythropoiesis, other studies show that mTOR inhibition may have beneficial effects in some forms of anemia including SCD and thalassemia, again emphasizing context- or disease-dependent functions for this pathway. Our mouse model makes it possible to investigate further the roles of miR-144/451, including its effects on mTOR, in physiological adaptations to various red cell disorders.

miR-144/451 is a bicistronic gene whose expression is directly controlled by GATA1 in erythroid cells. Interestingly, our research and that of others had previously observed that: 1) miR-144 level is always lower than miR-451 level in both fetal and adult erythroid cells; and 2) the expression of miR-44 is ubiquitous whereas the expression of miR-451 is much more constrained in hematopoietic compartments during embryonic development. These data suggest that overlapping and independent mechanisms regulate the differential expression of miR-144 and miR-451. The current study focuses on an miR-451-dependent mechanism for regulating stress erythropoiesis. However, it is not clear whether miR-144 impacts this process. Of note, suppression of miR-44 inhibits erythropoiesis in cultured human CD34+ cells. In future studies, it will be interesting to investigate the effects of miR-144 and miR-451 on erythropoiesis separately by examining mice that harbor single miR-specific mutations.

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