The miR-144/451 locus is required for erythroid homeostasis

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The process of erythropoiesis must be efficient and robust to supply the organism with red blood cells both under condition of homeostasis and stress. The microRNA (miRNA) pathway was recently shown to regulate erythroid development. Here, we show that expression of the locus encoding miR-144 and miR-451 is strictly dependent on Argonaute 2 and is required for erythroid homeostasis. Mice deficient for the miR-144/451 cluster display a cell autonomous impairment of late erythroblast maturation, resulting in erythroid hyperplasia, splenomegaly, and a mild anemia. Analysis of gene expression profiles from wild-type and miR-144/451–deficient erythroblasts revealed that the miR-144/451 cluster acts as a “tuner” of gene expression, influencing the expression of many genes. MiR-451 imparts a greater impact on target gene expression than miR-144. Accordingly, mice deficient in miR-451 alone exhibited a phenotype indistinguishable from miR-144/451–deficient mice. Thus, the miR-144/451 cluster tunes gene expression to impart a robustness to erythropoiesis that is critical under conditions of stress.
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abundantly expressed, but completely absent in Ago2−/− erythroblasts (Fig. 1, A and B). This is in stark contrast to other miRNAs that were down-regulated but still expressed. The gene encoding miR-451 is located on chromosome 11, and encompasses a noncoding transcriptional unit that also encodes miR-144 that resides 5′ of miR-451 (Fig. S1 A). Mir-144 was also abundantly expressed in erythroblasts and markedly down-regulated in the absence of Ago2 (Fig. 1, A and B). Expression analysis of both miR-451 and miR-144 within lineage-restricted lymphocytes, granulocytes/monocytes, and erythroid cells revealed that the locus was selectively expressed within the erythroid lineage (Fig. 1 C; Lu et al., 2008). The expression of miR-451 was restricted to the fetal liver in embryonic day (E) 16.5 mouse embryos, the major site of hematopoiesis and erythropoiesis at this stage of cell (Lim et al., 2005; Baek et al., 2008; Selbach et al., 2008), indicating that miRNAs not only function as developmental switches, but also as tuners of gene expression (Baek et al., 2008; Selbach et al., 2008).

RESULTS AND DISCUSSION

miR-144/451 expression in erythroblasts depends on Ago2

Recent studies revealed an essential role for Ago2 and the miRNA pathway during mammalian erythroid differentiation (O’Carroll et al., 2007). To identify miRNA loci that contribute to terminal erythropoiesis we used the following set of criteria: expression of the candidate miRNAs should be (a) down-regulated substantially in the absence of Ago2, (b) abundant in erythroblasts, and (c) restricted to the erythroid lineage. Expression profiling revealed that miR-451 was abundantly expressed, but completely absent in Ago2−/− erythroblasts (Fig. 1, A and B). This is in stark contrast to other miRNAs that were down-regulated but still expressed. The gene encoding miR-451 is located on chromosome 11, and encompasses a noncoding transcriptional unit that also encodes miR-144 that resides 5′ of miR-451 (Fig. S1 A). Mir-144 was also abundantly expressed in erythroblasts and markedly down-regulated in the absence of Ago2 (Fig. 1, A and B). Expression analysis of both miR-451 and miR-144 within lineage-restricted lymphocytes, granulocytes/monocytes, and erythroid cells revealed that the locus was selectively expressed within the erythroid lineage (Fig. 1 C; Lu et al., 2008). The expression of miR-451 was restricted to the fetal liver in embryonic day (E) 16.5 mouse embryos, the major site of hematopoiesis and erythropoiesis at this stage of

Figure 1. Expression of miR-144/451 locus is dependent on Ago2 and restricted to the erythroid lineage. (A) The levels of miRNA expression in sorted Ago2fl/fl and Ago2−/− erythroblasts (Ter119hiCD71hi) were measured by miRNA array profiling. Graph shows expression, normalized to the housekeeping genes RNU6A, RNU6B, SNORD2, and SNORD3A, of miRNAs displaying >1.5-fold down-regulation in Ago2−/− erythroblasts. The mean and standard deviation from three independent experiments are shown. (B) Expression of miR-451 and miR-144 in erythroblasts was quantified by qRT-PCR and graphs show expression normalized to U6 snRNA. The mean and standard deviation of two independent measurements are shown. (C) Expression of miR-144 and miR-451 quantified by qRT-PCR in erythroid (E), lymphoid (B/T), and granulocyte/monocyte cells (G/M). The mean and standard deviation of two independent measurements are shown. (D) The spatial expression of miR-451 is shown by in situ hybridization on sagittal sections of E16.5 dpc mouse embryos; hybridization with a control scrambled probe is also shown. Arrow indicates miR-451 expression in the fetal liver (FL). Representative images from one of three independent experiments are shown. (E) The expression of miR-451 and miR-144 was measured by Northern blotting of RNA derived from a fetal liver culture under conditions that promote proliferation (P) or differentiation. Numbers show hours in differentiation conditions. U6 snRNA was used as a loading control. The Northern was performed once from a representative fetal liver culture.
development (Fig. 1 D). Furthermore, both miR-451 and miR-144 were expressed in fetal liver–derived erythroid cultures under media conditions that promote proliferation or terminal differentiation (Fig. 1 E). Indeed, consistent with our identification of miR-144/451 as putative miRNAs required for terminal erythropoiesis, the miR-144/451 locus was recently identified as a target gene of the essential erythroid transcription factor Gata1 (Dore et al., 2008).

MiR-144/451 deficiency results in erythroid hyperplasia, ineffective erythropoiesis, and a mild anemia

MiR-451 and miR-144 met all our criteria for miRNAs with a key function during mammalian terminal erythropoiesis; therefore, we proceeded to generate a mouse miR-144/451 cluster-null allele (miR-144/451−/−) by deleter-Cre–mediated (Schwenk et al., 1995) excision of the loxP-flanked miR-144/451 allele (Fig. S1 B). MiR-144/451−/− mice were born in Mendelian numbers, survived throughout adulthood without increased rates of mortality, and showed no developmental defects in the lymphocyte or granulocyte-monocyte lineages (unpublished data). However, analysis of adult hematopoiesis in miR-144/451−/− mice revealed defects during terminal erythropoiesis. Peripheral blood showed a mild anemia with reduced red cell numbers and hematocrit and hemoglobin values, as well as increased red cell distribution width, indicating anisocytosis (Fig. 2 A and Fig. S2 A). The miR-144/451−/− mice also displayed splenomegaly, indicating compensation of anemia by stress erythropoiesis (Fig. 2 B). The anemia and stress erythropoiesis was confirmed by elevated erythropoietin concentrations (Fig. 2 C) and increased numbers of splenic c-kit–positive erythroid precursors in miR-144/451−/− mice (Fig. S2, B and C). Analysis of erythroid development revealed ineffective erythropoiesis in the bone marrow of miR-144/451−/− mice characterized by the normal presence of early basophilic erythroblasts (Ter119hi CD71hi), but a marked decrease in the frequency and number of mature orthochromatophilic (Ter119hi, CD71neg) erythroblasts (Fig. 2, D and E). In the mouse, inefficient erythropoiesis in the bone marrow can be compensated by stress erythropoiesis in the spleen.

**Figure 2.** MiR-144/451 deficiency results in erythroid hyperplasia, ineffective erythropoiesis, and anemia. (A) MiR-144/451 deficiency alters several erythrocyte parameters in the peripheral blood. Bar graphs are shown expressing values for RBC, hemoglobin (Hb), hematocrit (HCT); and red cell distribution width (RDW). Bars represent mean values (n = 5), error bars indicate standard deviation. (B) Splenomegaly in miR-144/451−/− mice. Bars represent mean values (n = 5), error bars indicate standard deviation. (C) Erythropoietin (EPO) concentrations in plasma in miR-144/451−/− and wild-type littermate controls. The individual and mean EPO values are plotted for five independent measurements. (D) Representative FACS analysis of erythroid cell populations in the bone marrow and spleen of wild-type and miR-144/451−/− mice. Roman numerals and numbers indicate the identity and percentages of cells of the developmentally defined subpopulations (Socolovsky et al., 2001): I, pro-erythroblasts; II, basophilic erythroblasts; III, polychromatophilic erythroblasts; IV, orthochromatophilic erythroblasts. (E) Comparative enumeration of erythroid precursors in the bone marrow and spleen of miR-144/451−/− and wild-type mice. Numbers are expressed relative to the wild-type and plotted for the developmentally defined erythroid subpopulations indicated in D by roman numerals. The data in A, B, D, and E are representative of three independent experiments. (F) Survival plot of wild-type (n = 11) and miR-144/451−/− (n = 11) mice treated with phenylhydrazine. As indicated by the arrows, days 0 and 1 mark the days of the first and second injection, respectively. The data represent two independent experiments. *, P < 0.05; **, P < 0.0001.
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The mild anemia in resting miR-144/451−/− mice may underrepresent the importance of the miR-144/451 locus to the hematopoietic homeostasis under situations of stress. We therefore challenged wild-type and miR-144/451−/− mice with phenylhydrazine, a chemical that induces hemolytic anemia, and assayed the potential of the mice to recover. Over half of the miR-144/451−/− mice died within 6 d after the administration of phenylhydrazine, whereas all wild-type littermates made a full recovery (Fig. 2 F). These results indicate that the miR-144/451 locus is critical for a robust hematopoietic system.

The defects in miR-144/451−/− mice are erythroid autonomous

Although unlikely, one could argue that the defects observed in the miR-144/451−/− mice are not hematopoietic autonomous and that they do not represent a developmental defect, but are a consequence of the response to a hemolytic anemia. The hematopoietic-intrinsic nature of the developmental defects in...
miR-144/451−/− mice was revealed through the analysis of lethally irradiated mice reconstituted with miR-144/451−/− bone marrow. These reconstituted mice present the same defects as germ-line-deficient miR-144/451−/− mice (Fig. S3). We next used an in vitro fetal liver–derived erythroid cell culture and differentiation system (Dolzgin et al., 2001; von Lindern et al., 2001) to investigate the intrinsic nature of the developmental defect. Fetal livers from wild-type and miR-144/451−/− E12.5 embryos were placed in culture with a cocktail of defined cytokines that promote the selective expansion of erythroid precursors. Under these conditions miR−144/451−/− erythroid precursors proliferated at the same rate as the respective wild-type controls (Fig. 3 A). After 3 d of expansion, the cultures were switched to differentiation medium. MiR-144/451−/− cultures normally underwent the final rounds of proliferation before entering terminal erythropoiesis (Fig. 3 B), as well as hemoglobinization (Fig. S2 E). In addition, no differences in the fraction of apoptotic cells were detected in the absence of miR-144/451 (Fig. 3 C). However, size reduction and nucleation of miR-144/451−/− cultures was inefficient (Fig. 3, D and E, and Fig. S2 E). MiR-144/451 deficiency did not block any one process, per se, but rendered terminal erythroid differentiation inefficient. Thus, the in vitro system recapitulated the defective erythropoiesis observed in vivo in miR-144/451−/− mice. These observations prove that the miR-144/451 cluster is not required for expansion of early erythroblasts, but is intrinsically required for terminal erythroid development.

The miR-144/451 locus tunes the expression of many genes

We next wanted to explore the extent and nature of the deregulated genes in the absence of miR-144/451 during terminal erythropoiesis. To this end, we decided to use the in vitro culture model, as it presented advantages over ex vivo isolated erythroblasts. The miR-144/451−/− erythroblasts in vivo are subjected to, among other things, altered cytokine levels and microenvironments. We detected a significant deregulation of gene expression in miR-144/451−/− erythroblasts (Fig. 4 A). Transcriptome analysis from cells with either loss or gain of function for a given miRNA has been used to reliably identify miRNA targets because of transcript destabilization after RISC binding (Lim et al., 2005; Giraldes et al., 2006; Baek et al., 2008; Selbach et al., 2008; Lewis et al., 2009). To identify miR-144/451 target genes, we therefore used the Sylamer algorithm (van Dongen et al., 2008) to analyze the gene expression profiles derived from wild-type and miR-144/451−/− erythroid precursors. Sylamer searches for significant enrichment of 6–8 nt motifs across an ordered gene list. For miRNA analysis, the identification of miRNA seed match enrichment in the 3′ UTRs of deregulated genes from genome-wide experiments indicates direct miRNA involvement (van Dongen et al., 2008).

Figure 4. The miR-144/451 locus tunes the expression of many genes. (A) Expression scatterplot showing relative mean expression of Illumina probes in wild-type (x axis) and miR-144/451−/− (y axis) erythroblasts. Significantly up-regulated genes (red) and down-regulated genes (green) are shown. Expression profiling was performed once with biological quadruplicates of each genotype. (B) Sylamer enrichment landscape plot for all 876 7-nt motifs complementary to canonical mouse miRNA seed regions, either from nt 2–8 (7mer-m8) or from nt 1–7 (7mer-A1). The x axis represents the sorted gene list of 19,600 genes from most up-regulated to most down-regulated in the miR-144/451−/− erythroblasts. Each 7mer motif was tested for significant enrichment across the 3′ UTRs of genes in this list. The two motifs corresponding to seeds matching miR-451 (red, 7mer-m8; yellow, 7mer-A1) are enriched in the up-regulated genes, peaking at the most up-regulated 588 genes (solid vertical line). Two motifs matching the seed region of miR-144 (blue, 7mer-m8; light blue, 7mer-A1) are also significantly enriched, but to a lesser extent. The horizontal dotted lines represent a Bonferroni-corrected P value threshold of 0.005. (C) Upper region of an empirical cumulative density plot of log fold change for different populations of probes. Those probes whose 3′ UTRs possess miR-144 6-nt seed matches (green), miR-451 6-nt seeds (orange), or both seeds (red) are skewed toward higher positive fold changes in the miR-144/451−/− erythroblasts, compared with probes whose mapped 3′ UTRs do not contain a seed match (black). (D) qRT-PCR expression analysis of representative miR-144/451 seed-containing deregulated genes identified by Sylamer. Normalized data are plotted as relative fold change in miR-144/451−/− versus wild-type erythroblasts. Standard error is shown and * indicates significantly up-regulated expression (P < 0.05). Genes are color coded as in C. Data were obtained in two independent experiments measuring biological quadruplicates of each genotype.
Sylamer analysis using all 7-nt motifs complementary to seed regions of mouse miRNAs revealed a highly significant (P < 8 × 10^{-13}; Bonferroni corrected < 7 × 10^{-10}) enrichment for the 7-nt seed match of miR-451 (AACGGTT) in the 3′ UTRs of genes in a cohort of 588 genes, most of which were up-regulated in miR144/451−/− erythroid precursors (Fig. 4 B). Enrichment of the seed sequence of miR-144 (ATACGTG) was observed, but was less significant (P < 4 × 10^{-6}, Bonferroni corrected < 4 × 10^{-3}). Genes with 6-nt seed matches for miR-144 were far less affected than those containing miR-451 sites (Fig. 4 C). However, genes containing 6-nt seed matches for both miR-144 and miR-451 displayed a slight additive effect and were more deregulated than genes with miR-451 seed matches alone. The deregulation of a representative panel of the Sylamer-identified miR-144/451 seed-containing genes was validated by quantitative RT-PCR (qRT-PCR; Fig. 4 D). Of the 588 up-regulated genes identified by Sylamer analysis in the miR-144/451−/− erythroblasts, 98 had one or more 6-nt seed matches for miR-451, 226 had seed matches for miR-144, and 57 of these contained seed matches for both (Table S1). Many of these genes have reported roles in terminal erythropoiesis (Table S1), but the majority are loci whose precise expression are only now linked to normal erythroid development (Table S1). This analysis is not suggestive of a single, or few critical miR-144 or miR-451 targets, but rather that the miR-144/451 locus regulates many genes whose collective deregulation derails normal terminal erythropoiesis. We did not find evidence of lineage-inappropriate gene expression, excluding the possibility that the miR-144/451 cluster functions to maintain lineage identity. Together, these data suggest that the miR-144/451 locus functions as a tuner of gene expression, analogous to the function of miR-223 in neutrophils (Baek et al., 2008).

MiR-451 regulates terminal erythropoiesis

Many of our observations suggest that within the miR-144/451 cluster, miR-451 is likely to play the pivotal role in terminal erythropoiesis. First, it displays a far higher expression level than miR-144. Second, our bioinformatics analyses showed a greater statistical enrichment for the miR-451 seed in the up-regulated cohort of genes in the miR-144/451 mutants. Third, miR-451 exerted a greater impact on the level of target gene expression than miR-144. To explore the possibility that miR-451 plays a key role in regulating terminal erythropoiesis, we generated and analyzed miR-451-null (mir-451−/−) mice (Fig. S1 C). The phenotype of miR-451−/− mice was largely indistinguishable from that of miR-144/451−/− mice (Fig. 5, A–D). Together, these results indicate that deregulation of miR-451 targets is sufficient to impair erythroid development.

In this study, we show that miR-451 expression is strictly dependent on Ago2 function. Indeed, while this manuscript was under review it was shown that biogenesis of miR-451 is thus far unique in that it is Dicer independent but requires the endonuclease activity of Ago2 (Cheloufi et al., 2010; Chilueta et al., 2010). The phenotype of miR-144/451−/− and miR-451−/− mice is milder than that of Ago2−/− mice (O’Carroll et al., 2007), indicating that additional miRNA loci regulate terminal erythropoiesis. In addition, the viability of miR-451−/− mice suggests that the perinatal lethality in homozygous

Figure 5. Selective disruption of miR-451 within the miR-144/451 locus results in erythroid hyperplasia, ineffective erythropoiesis, and anemia. (A) MiR-451 deficiency alters several erythrocyte parameters in the peripheral blood. Bar charts are shown expressing values for red blood cell (RBC) numbers, hemoglobin (Hb), hematocrit (HCT); and red cell distribution width (RDW). Bars represent mean values (n = 4), error bars indicate standard deviation. (B) Splenomegaly in miR-451−/− mice. Bars represent mean values (n = 4), error bars indicate standard deviation. (C) Representative FACS analysis of erythroid cell populations in the bone marrow and spleen of wild-type and miR-451−/− mice. (D) Comparative enumeration of erythroid precursors in the bone marrow and spleen of wild-type and miR-451−/− mice. Bars represent mean values (n = 4), error bars indicate standard deviation. (C) Representative FACS analysis of erythroid cell populations in the bone marrow and spleen of wild-type and miR-451−/− mice. (D) Comparative enumeration of erythroid precursors in the bone marrow and spleen of wild-type and miR-451−/− mice. For B and C, data are represented as in Fig. 5. A–D. #, P < 0.05; **, P < 0.01. The data in all panels are from biological quadruplicates and representative of two independent experiments.
endonuclease-dead Δg02A40H mutant mice cannot be accounted for solely by the loss of miR-451 expression. Analogous to the granulocytic and the lymphoid lineages, we now show that a miRNA-encoding locus regulates erythroid development in vivo. We demonstrate that loss of miR-144/451 or miR-451 does not block any one process, but renders terminal erythroid differentiation inefficient. We show that the miR-144/451 cluster tunes the expression of many genes. Gata1 confers the erythroid lineage-specific expression of miR-144/451 (Dore et al., 2008). Therefore, Gata1 not only ignites the program of terminal erythroid differentiation, but also ensures that gene expression is tuned through the activation of the miR-144/451 locus.

**MATERIALS AND METHODS**

**MiRNA expression analysis.** RNA was isolated using Trizol (Invitrogen) according to manufacturer’s instructions. 500 ng of total RNA was labeled and hybridized to miCURry LNA microRNA arrays V.11 (Exqon) for miRNA profiling. Array Express accession no. E-TABM-996. qRT-PCR were performed using TaqMan miRNA assays (Applied Biosystems) for miR-144 and miR-451 and miRNA LNA U6, respectively. In situ hybridizations were performed on cryosections with specific LNA probes (Exiqon).

**Generation of miR144/451- and miR-451-null alleles.** The MiR-144/451 targeting strategy allows Cre-mediated deletion of transcription start site (TSS) for the miR-144/451 cluster, as well as the hairpin that encodes miR-451. This deletion simultaneously deletes miR-144 and transcription initiation of the cluster, leading to the loss of both miR-144 and miR-451 expression. The miR-144/451 gene targeting vector (pDTA-miR-144/451) carries a neomycin resistance (neo) gene flanked with two frt sites and a loxp site (neo-frt2-loxp) cassette located 3’ of the TSS and a second loxp site between miR-144 and miR-451.

The MiR-451 targeting strategy allows Cre-mediated deletion of the hairpin that encodes miR-451. This deletion leaves the TSS and miR-144 transcription intact, resulting in the loss of miR-451 expression. The miR-451 gene targeting vector (pDTA-miR-451) carries a loxp site between miR-144 and miR-451 and a neomycin resistance (neo) gene flanked with two frt sites and a loxp site (miR-451-neo-frt2-loxp) cassette located 3’ of the hairpin that encodes miR-451.

The targeting constructs were transfected into IB10 ES cells. Southern blotting of the individual ES cells clones was used to identify homologous recombination. Targeted ES cells were used to generate mice heterozygous for the miR-144/451– and miR-451– targeted alleles. These mice were then crossed to the FLP-expressing transgenic mice (FLPeR) to remove the FLP vector. Targeted ES cells were used to generate mice heterozygous for miR-144/451 and miR-451 loxP sites, respectively. In situ hybridizations were performed on cryosections with specific LNA probes (Exqon).

**Gene expression data analysis.** Live proliferating erythroblasts (FSC-Aomed from eight independent erythroid cultures (four wild-type and four miR-144/451−/−) was sorted by FACS to obtain highly homogeneous cell populations for gene expression profiling. Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer’s instructions and profiled using Illumina mouse WG-6 v2.0 Bead arrays. Probe level data from all eight arrays were obtained from the Illumina GenomeStudio software and processed using R/BioConductor using a variance stabilizing transformation and robust spline normalization as implemented in the limma package (Du et al., 2008). Differential expression between wild-type and miR-144/451−/− samples was assessed by fitting linear models and using an empirical Bayes approach to shrink the estimated variance, by means of the limma package (Smyth, 2004). Probe annotations and mappings were obtained from Illumina (MouseWG-6_V2_0_R2_11278593_A). The gene expression data are deposited in Array Express under the accession no. E-MTAB-277.

**Quantitative real-time PCR.** Total RNA was reverse transcribed by Superscript II reverse transcription (Invitrogen) using random hexamer primers. Quantitative PCR was performed on a LightCycler 480 PCR instrument (Roche) using 2x SYBR green I master (Roche). The expression data were normalized to Hprt and Pdgfβ reference genes and analyzed by REST software (Pfaffl et al., 2002) using pairwise fixed reallocation and randomization test to estimate relative normalized fold change between wild-type and miR-144/451−/− erythroblast samples.

**Sylamer analysis.** The gene-expression data for each probe were sorted according to t-test statistic from most significantly up-regulated to most significantly down-regulated in the knockouts samples. Each probe ID was mapped to a mouse transcript, and then to a 3’ UTR sequence. The 3’ UTR sequences were obtained by mapping probes to transcript sequences from Vega, Ensembl, or RefSeq, in that order of preference. An ordered list of 19,600 unique transcripts was produced in this fashion, each with a corresponding 3’ UTR sequence. Sylamer was run on this dataset with Markov correction set to filter trinucleotide biases and analyzing enrichment of 7–nt motifs.

**Online supplemental material.** Fig. S1 illustrates and validates the generation of the miR-144/451 and miR-451 null alleles, respectively. Fig. S2 depicts histological analysis of peripheral blood, BM, and spleen of

**Phenylhydrazine treatment.** Adult male mice were administered with phenylhydrazine at a dose of 48 mg/kg by intraperitoneal injection on two successive days, as previously described (Gutiérrez et al., 2008).

**Erythroid in vitro culture system.** The in vitro expansion and differentiation of erythroid cells from E12.5 fetal livers was performed as previously described (Dolznig et al., 2001; von Lindern et al., 2001). Cell size reduction during differentiation was indirectly assessed by FSC-A using FACS analysis. To assess morphology, cells were spun onto glass slides, air dried, and stained with o-dianisidine (Neutral Benzidine; Sigma-Aldrich) in 1% methanol and Diff Quick staining set (Medion Diagnostics AG). Images were acquired with a microscope at 63×, 1.30 numerical aperture oil immersion lens fitted with a DFC290 camera and processed with Adobe Photoshop. The appearance of nascent reticulocytes was enumerated using a modified FACS protocol as described (Kingley et al., 2006). Instead of Hoechst 33342, cells were stained for 30 min at 37°C with 20 μM Vybrant Violet (Invitrogen). CFSE labeling and dilution was used to monitor the number of cell divisions upon terminal differentiation as described (Gutiérrez et al., 2008). Annexin V FACS staining (Annexin V apoptosis detection kit; ebioscience) was used to enumerate the number of apoptotic cells in the cultures.
miR-144/451-deficient mice, as well as FACS analysis enumerating erythroid precursors. Morphological analysis of in vitro erythroid cultures is also shown. Fig. S3 contains the analysis of lethally irradiated mice reconstituted with miR-144/451-/- bone marrow. Table S1 lists the Sylamer-identified target genes of miR-144/451. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20100458/DC1.

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REFERENCES


SUPPLEMENTAL MATERIAL

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Figure S1. Generation of miR–144/451 and miR–451 null alleles. (A) Overview of the miR–144/451 locus at high resolution (top). The organization of the miR144/451 locus and adjacent Era1 gene that lies on the opposite strand is depicted (bottom). The EcoRI (E) and AflII (A) restriction sites are indicated as the well as the respective Southern fragments detected by probe A. Position of the DNA encoding the premiR–144 and premiR–451 are indicated in red and blue, respectively. The filled rectangles represent exons and the unfilled rectangle represents the 3’UTR of Era1. (B) Targeting strategy and validation of the miR–144/451 null allele. The targeting vector used for introduction of loxP sites into the miR144/451 locus and the schematic map of the targeted miR144/451 before and after, Flp and Cre-mediated-recombination are shown (left). Shaded triangles represent loxP sites and frt sites as indicated. Shaded rectangles indicate the position of Neomycin (Neo) and Diptheria toxin A (DTA) selection marker genes. The EcoRI (E) restriction sites are indicated in the miR–144/451 null allele as the well as the respective Southern fragment detected by probe A. Deletion diagnosed by Southern blotting of tail derived EcoRI-digested DNA is presented (right panel). The absence of miR–451 and miR–144 in bone marrow determined by Northern blotting is shown. The miRNA Let–7a expression is shown as a loading control. The data are representative of two independent experiments. (C) Targeting strategy and validation of the miR–451 null allele. The targeting strategy for the inactivation of miR–451 is depicted (left panel) as in (B). The AflII (A) restriction sites are indicated in the miR–451 null allele as the well as the respective Southern fragment detected by probe A. Deletion diagnosed by Southern blotting of tail derived AflII-digested DNA is presented (right panel). The selective absence of miR–451 in bone marrow determined by Northern blotting is shown. U6 expression is shown as a loading control. The data are representative of two independent experiments.
Figure S2. Characterization of miR–144/451−/− erythroid cells. (A) Modified Wright-stained peripheral blood smears from wild-type and miR–144/451−/− mice. Mutant erythrocytes show variation in size and shape (anisocytosis) as well as increased levels of young erythrocytes (blue shade) in circulation. Representative images from five independent experiments are shown. (B) Representative FACS analysis is shown for the c-kit-positive erythroid cell populations in the bone marrow and spleen of wild-type and miR–144/451−/− mice. The gates illustrate the following developmentally defined subpopulations: pre–colony-forming unit erythroid precursor (Pre-CFU-E), colony forming unit erythroid (CFU-E), pre–megakaryocyte/erythroid precursor (Pre-MegE), granulo monocyte precursor (GMP), and pregranulo monocyte precursor (Pre-GM). (C) Comparative enumeration of lineage-negative erythroid precursors in the bone marrow and spleen of wild-type and miR–144/451−/− mice. Numbers are expressed relative to the wild-type and plotted for the indicated subpopulations. The data are from biological quadruplicates and representative of two independent experiments. A single asterisk represents significance of P < 0.01. (D) Histological analysis of wild-type (upper panel) and miR–144/451−/− (lower panel) erythropoietic tissues by H&E staining. Architecture and morphology of bone marrow (left) and spleen (right) are shown. miR–144/451-deficient spleen shows a pronounced increase in the density of clusters of maturing erythroblasts, whereas no marked morphological differences are observed in bone marrow. Images are representative of 4 independent experiments. (E) Diff-Quick-Benzidine–stained cytospins of in vitro erythroid cultures derived from wildtype (left) and miR–144/451−/− (right) mice at the indicated time points during differentiation. Brown staining indicates presence of hemoglobin. Thin arrows indicate nascent reticulocytes and thick arrows indicate nucleated erythroblast. Images are representative of four independent experiments. Bars: (A) 10 μm; (D) 40 μm; (D, inset) 20 μm; (E) 10 μm.
Figure S3. The erythroid defects in miR-144/451−/− mice are hematopoietic autonomous. (A) Representative FACS analysis is shown of erythroid cell populations in the bone marrow and spleen of lethally irradiated mice reconstituted with wild-type or miR-144/451−/− bone marrow, as indicated. Mice were analyzed 12 wk after reconstitution. Numbers indicate the percentages of cells of the developmentally defined subpopulations. The roman numerals indicate the identity of the following defined erythroid subpopulations (Socolovsky et al., 2001): I, proerythroblasts; II, basophilic erythroblasts; III, polychromatophilic erythroblasts; and IV, orthochromatophilic erythroblasts. (B) Comparative enumeration of erythroid precursors in the bone marrow and spleen of reconstituted mice. Genotype of donor bone marrow is indicated. Numbers are expressed relative to the wild-type and plotted for the developmentally defined erythroid subpopulations indicated in (A) by roman numerals. (C) MiR-144/451 deficiency alters several erythrocyte parameters in the peripheral blood. Bar charts are shown expressing values for RBC, hemoglobin (Hb), hematocrit (HCT); and red cell distribution width (RDW). Bars represent mean values (n = 4), error bars indicate standard deviation. The data in all panels are from biological quadruplicates and representative of two independent experiments. *P < 0.05; **, P < 0.01.