Erythrocyte survival is controlled by microRNA-142

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Mouse genetics, surgical procedures and husbandry

Mice strains were housed and handled in accordance with protocols approved by the Institutional Animal Care and Use Committee of WIS. To generate miR-142−/− mice, a gene-trapped embryonic stem cell clone (ES, C57BL/6J strain) from TIGM (College Station, TX), was chosen based on an insertion upstream of miR-142 hairpin. ES cells were microinjected into C57BL/6J host blastocysts. Chimeras and further transmission of the targeted allele through the male germline to heterozygous pedigree was confirmed by PCR analysis of genomic tail DNA. Homozygous and WT littermate mice were generated by additional intercrosses.

Phenylhydrazine was administered to adult male mice at a dose of 48 mg/kg by intraperitoneal injection, on two successive days, as previously described (20).

Hematologic and histology studies

100 μl whole blood was drawn via retro-orbital approach into glass capillary tubes that were pre-treated to prevent coagulation, with 5 μl of 0.5M EDTA. Complete blood count was performed on ADVIA 120 Hematology System (Siemens Healthcare, Erlangen, Germany) by American Medical Laboratories (Herzliya, Israel). Reticulocytes were enumerated on peripheral blood (PB) smears stained with Methylene blue or May–Grünwald Giemsa. After euthanasia spleens and limbs were excised and fixed overnight in 4% paraformaldehyde. Femora were further decalcified in 14% EDTA for 2–5 days. Specimens were dehydrated in graded ethanols, washed and processed into paraffin blocks. Longitudinal paraffin sections were stained with hematoxylin and eosin (H&E).

Bright-field micrographs captured with Olympus BX51 microscope at 40X magnification.

Phenylhydrazine induced RBC lysis

Red blood cells were washed and re-suspended in PBS, incubated with various concentrations of PHZ for 4 h at 37ºC. Percentage of non-lysed RBCs was determined by Methylene Blue staining in glass hemocytometer.

ROS measurement

Red blood cells were washed and resuspended in PBS and labeled with Ter119 antibody for 30 minutes at 4°C, washed in pre-warmed PBS and loaded with 10µM of 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) in the dark for 30 minutes at 37°C and 5% CO2. Cells were washed with pre-warmed PBS and sorted using fluorescence activated cell sorter (FACS) with LSRII instrument. Data were analyzed using Cell Quest (BD Bioscience).

RBC life span

Mice were injected with 1 mg of EZ-Link Sulfo-NHS-Biotin (Thermo Scientific) in 300 µL of PBS, and percentages of circulating biotinylated RBCs were analyzed by FACS (LSRII instrument) after staining with streptavidin- Cy5 and Ter119 antibodies.

Electron microscopy

100 µl whole blood was gently washed in Cacodylate buffer (0.1 M), fixed with 3% paraformaldehyde and 2% gluteraldehyde in Cacodylate buffer (0.1 M) overnight at 4°C, washed with Cacodylate buffer (0.1 M) and placed overnight at 4^{o}C on silica chip that were freshly coated with poly-L-lysine. The next day, samples were stained with 1% OsO4 for 1h in RT, washed several times with DDW, dehydrated in graded ethanols (30%, 50%, 70%, 96 and 100%). Then, the samples were dried in the CPD 030 (BAL-TEC), sputtered in gold palladium sputter coater (EDWARDS) and visualized using secondary electron (SE) detector in high-resolution Ultra 55 (Zeiss) or ESEM (FEI) microscopes.

Super resolution microscopy

Peripheral blood (50 µL) was diluted in 1 ml PBS, erythrocytes were fixed with 3% paraformaldehyde and 2% gluteraldehyde for 30 min, washed with PBS and placed overnight on poly-L-lysine (1%) coated coverslips (MatTek P35G-1.5-14-C). The next day, erythrocytes were permeabilized in PBS, 0.1% Triton X-100 for 5 min rinsed 3 times in PBS and stained with Alexa647-conjugated Phalloidin (A22287, Life technologies) 1:500. Coverslips submerged in Petri dishes, filled with imaging buffer (7 µM glucose oxidase (Sigma), 50 mM cysteamine (Sigma), 50 mM Tris, 10 mM NaCl, 56 nM catalase (Sigma), 10% glucose, pH 8). Micrographs captured on Vutara SR200 STORM microscope with 647 nm laser excitation power of about 7 kW/cm2. Z-stack image was measured by acquiring 700 frames at 50 Hz for each z position of 0.1 um steps, and 405-nm activation laser power was ramped slowly to

maintain optimal single-molecule density. Single-molecule fitting was performed with Vutara software.

RBC Deformability

RBC deformability was determined using a computerized cell flow-properties analyzer (CFA), designed and developed at the Yedgar lab (21, 22). CFA is containing a narrow-gap flow- chamber (adjustable to 10-200 μm gap), in which the change in the RBC shape is visually monitored as a function of flow-induced shear stress. 50 μl of RBC (1% hematocrit, in GPBS) are placed on and adhere to an un-coated polystyrene slide (Electron Microscopy Science, Washington, PA, USA) in the flow-chamber, and subjected a controllable flow-induced shear stress. The cell shape-change is expressed by the change in the cell axial ratio, namely the elongation ratio, ER = a/b, where a/b = the major/the minor cell axes (ER = 1 for a perfectly round erythrocyte). The image analysis provides the ER distribution in a large RBC population (at least 2,500 ± 300 cells), from which different deformability parameters (average, median ER, or any respective percentile) can be derived. As the axes measurement accuracy is about 10%, cells with ER ≤ 1.1 are defined as 'undeformable' RBC, namely cells that do not deform under high shear stress (3.0 Pa). Average erythrocyte elongation ratio (WT = 1.85% ± 0.073; miR-142-/- = 2.01% ± 0.114; P = 0.027). Percent of undeformed cells, with elongation ratio ≤1.1: WT = 2.4% ± 1.63; miR-142-/- = 1.33% ± 1.58; P = 0.027. Percent of highly-deformed cells, with elongation ratio ≥2.5: WT = 5.33% ± 2.94; miR-142-/- = 11.16% ± 3.15; P = 0.020.

RBC osmotic fragility

Osmotic fragility was determined, following (22, 23). 10 µl of whole blood are mixed with 1 ml NaCl solution at varying concentrations (0.0 g/l, 0.2 -0.9 g/l at 0.1 g/l steps). Cells were incubated at room temperature for 1 h, and centrifuged at 500 × g for 10 min. Hemolysis was determined by free hemoglobin optical density (OD) at 540 nm in the supernatant. NaCl concentration of, exerting 50% hemolysis, [NCl]50%, (WT = 0.525%± 0.003; miR-142-/- = 0.45%± 0.02; P = 0.0029). Maximal NaCl concentration, [NCl] max, (WT = 0.5%; miR-142-/- = 0.4%; P < 0.001). Slope factor of cumulative hemolysis curve, SF, (%-1), (WT = 369%-1± 25 ; miR-142-/- = 384%-1± 13).

Western Blot

Freshly heparinized blood was collected from wild-type and miR-142-/- mice. Blood samples were centrifuged at 1,000 g for 10 min. The plasma and buffy coat were removed by aspiration. Washed RBCs were then collected and lysed in 25 mM Tris_HCl, pH 7.6, 150 Mm NaCl, 1% Triton X-100, 1% P-40, 1% sodium deoxycholate, 0.1% SDS, supplemented with 1 protease inhibitor cocktail tablet per 10 mL (0.15 ml of lysis solution per 1X107 cells). 20µg of proteins were separated by SDS polyacrylamide gel electrophoresis, electrotransferred onto 0.2mm nitrocellulose membrane, blocked in TBS, 0.1% Tween20 and 5% dry milk for 1h and incubated overnight with primary Antibodies: anti-N-WASP / Wasl (4848s; Signaling Technology, 1:1,000), anti-Cofilin2 (ab96678; Abcam, 1:1,000), anti- GAPDH (AM4300; Ambion, 1:10,000), anti-Spectrin (ab11182; Abcam, 1:1,000), anti-Adducin (ab40760; Abcam, 1:1,000), anti-Band-3 (ab104998; Abcam, 1:1,000), anti-Ankyrin (ab58698; Abcam, 1:1,000) HRP-conjugated secondary

antibody (Jackson ImmunoResearch Laboratories) was diluted in TBS, 0.1% Tween20. Immunoreactive proteins were detected using ECL (GE Healthcare) and imaged using ImageQuant ˛ Las4010 (GE). Quantification of blots was performed using ImageJ imaging software.

K562 cells

K562 cells were cultured in RPMI 1640 (with 300 mg/l glutamine), supplemented with 10% fetal bovine serum and 1× antibiotics and antimycotics (100 U/ml ampicillin, 100 U/ml streptomycin) at 37 ºC in a 5% CO2 incubator. For stable transfection, K562 cells were washed twice with PBS and re-suspended in OPTI MEM (Invitrogen) at concentration of 1 × 10^6 cells/ml. For electroporation, 10^6 cells were mixed with 10µg miRZIP 142 plasmid (MZIP142-3p-PA-1-SBI, System Biosciences) or control GFP plasmid, incubated for 5 min at room temperature and electrotransfected using the BioRad electroporator (275 mV/1.5 ms). Cells were cultured for 3 days and then transfectants were selected by resistance to puromycin (2µg/ml) over a period of 14 days. Lentiviral production was performed as previously described (12). Viral transduction was performed through centrifugation of 50,000 cells/ 9 cm^2 (6-well) plate at 900×g, 32°C for 90 min with particles at multiplicity of infection of 25. For mock transduction, an equivalent volume of medium was added. Cells were incubated at 37°C for 3 days prior to analysis.

Fluorescence-activated cell sorting (FACS) and high-speed cell imaging analysis in flow using ImagestreamX

Isolation of bone marrow, FACS analysis, actin intensity and morphocytometry were performed as previously described (12). Cells were stained for PE-conjugated anti-CD71 antibody (transferrin receptor, Abcam, Cambridge, England), FITC-conjugated anti-Ter119 (Emfret), fixed with Cytofix/Cytoperm (BD Biosciences, San Jose, CA), and further stained with Alexa647-conjugated Phalloidin (A22287, Life technologies) and Hoechst. Enucleated mature erythrocytes express TER119 and lack CD71 and Hoechst, whereas reticulocyte precursors retain their nucleus and a re positiveily stained with CD71. Images were compensated for fluorescent dye overlap by using single-stain controls. Analysis was done on in-focus single cell images as previously described (24) with single cell gating. Cell area was calculated in square microns from bright-field images. Circularity was calculated as average distance of object boundary from center, divided by the variation of this distance. Thus, shapes approximating circle exhibited low variation and gained higher values (in arbitrary units). Actin polarity was calculated using the Delta Centroid XY feature as the distance (in microns) between erythrocyte bright-field image geometric center and the intensity-weighted actin image center (higher values indicate increased polarity). Position of actin in the cell was calculated using the Max Contour Position feature that calculates the location of the contour in the cell that has the highest intensity concentration. The actual location is mapped to a number between 0 and 1, where “0” indicates the center of the cell and “1” indicates the membrane of the cell. Approximately 100 000 events per experiment were collected and analyzed with the associated Image Data Exploration and Analysis software (IDEAS; Amnis).