Rac1 and Rac2 GTPases are necessary for early erythropoietic expansion in the bone marrow but not in the spleen

Theodosia A. Kalfa,[1,2] Suvarnamala Pushkaran,[2] Xiaoling Zhang,[2] James F. Johnson,[1] Dao Pan,[1] Deidre Daria,[1] Hartmut Geiger,[1] Jose A. Cancelas,[1] David A. Williams,[1,3] and Yi Zheng[1]

[1]Experimental Hematology and [2]Hematology/Oncology, Cincinnati Children's Research Foundation, Cincinnati Children's Hospital Medical Center and the University of Cincinnati College of Medicine, [3]Hematology/Oncology, Children's Hospital Boston, Harvard Medical School, USA

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Correspondence: Theodosia A. Kalfa, MD, PhD Division of Hematology/Oncology, Cincinnati Children's Hospital Medical Center 3333 Burnet Avenue, MLC 7015 Cincinnati, OH 45229-3039 E-mail: theodosia.kalfa@cchmc.org

ABSTRACT

Background

The small Rho GTPases Rac1 and Rac2 have both overlapping and distinct roles in actin organization, cell survival, and proliferation in various hematopoietic cell lineages. The role of these Rac GTPases in erythropoiesis has not yet been fully elucidated.

Design and Methods

Cre-recombinase-induced deletion of *Rac1* genomic sequence was accomplished on a *Rac2*-null genetic background, in mouse hematopoietic cells *in vivo*. The erythroid progenitors and precursors in the bone marrow and spleen of these genetically engineered animals were evaluated by colony assays and flow cytometry. Apoptosis and proliferation of the different stages of erythroid progenitors and precursors were evaluated by flow cytometry.

Results

Erythropoiesis in $Rac1^{-/-}$;$Rac2^{-/-}$ mice is characterized by abnormal burst-forming unit-erythroid colony morphology and decreased numbers of megakaryocyte-erythrocyte progenitors, erythroid colony-forming units, and erythroblasts in the bone marrow. In contrast, splenic erythropoiesis is increased. Combined Rac1 and Rac2 deficiency compromises proliferation of the megakaryocyte-erythrocyte progenitor population in the bone marrow, while it allows increased survival and proliferation of megakaryocyte-erythrocyte progenitors in the spleen.

Conclusions

These data suggest that Rac1 and Rac2 GTPases are essential for normal bone marrow erythropoiesis but that they are dispensable for erythropoiesis in the spleen, implying different signaling pathways for homeostatic and stress erythropoiesis.

Key words: Rho GTPases, deletion, Rac1 genomic sequence, erythropoiesis.

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Introduction

Mammalian erythropoiesis follows a defined series of steps leading from the first committed erythroid progenitors to enucleated red blood cells. During these processes the erythroid cells become committed, proliferate, and differentiate under the influence of cytokines directing tightly orchestrated signaling events. It has been well established that cytokines such as stem cell factor, interleukin-3, and erythropoietin, as well as the surrounding microenvironment, consisting of stromal cells, erythroblastic island macrophages, and extracellular matrix proteins such as fibronectin, play essential and non-redundant roles in erythropoiesis. However, the intracellular signaling mechanisms triggered by the various erythropoietic cytokines and by the microenvironment in order to regulate erythroid cellular responses remain poorly defined.[1-3]

Hematopoietic stem cells are first committed to either the lymphoid or myeloid lineage, differentiating into common lymphoid progenitors or common myeloid progenitors, respectively.[4,5] Common myeloid progenitors give rise to granulocyte-macrophage progenitors and megakaryocyte-erythrocyte progenitors (MEP).[4] MEP are characterized by flow cytometry as IL7Rα$^-$Lin$^-$cKit$^+$Sca1$^-$FcγR-II/III^{lo}CD34$^-$ cells.

The IL7Rα$^-$Lin$^-$cKit$^+$ cells have myeloerythroid colony-forming unit (CFU) activity. Excluding from this population the Sca1$^+$ cells, which are highly enriched for hematopoietic stem cells, and further focusing on the FcγR-II/III^{lo}CD34$^-$ fraction leaves the megakaryocyte/erythrocyte lineage-restricted progenitors, which in colony assays will give rise exclusively to erythroid burst-forming units (BFU-E), megakaryocyte CFU (CFU-Meg), and megakaryocyte/erythroid CFU (CFU-Meg/E) colonies generating only erythrocytes and megakaryocytes.[4] The early erythroid progenitors, which are defined either by colony assays as BFU-E and CFU-E, or by flow cytometry largely as a subset of MEP, proceed to further differentiation to the later erythroid precursors or erythroblasts. Morphologically, erythroblasts are classified into proerythroblasts, basophilic, polychromatophilic, and orthochromatic erythroblasts, depending on the morphology of the nucleus and the amount of hemoglobin in the cytoplasm, which affects the color of the cytoplasm of cells stained with Wright-Giemsa stain.[6] Erythroblasts and red blood cells carry a characteristic erythroid lineage surface marker, glycophorin-A, which can be labeled in mice by staining for the associated molecule Ter119.[7] In parallel CFU-E and erythroblasts in their various stages of maturation up to reticulocytes also carry transferrin receptor, CD71.[8] Flow cytometry results of erythroblasts after double staining for Ter119 and CD71 have been correlated to their morphology,[9-11] although with somewhat conflicting variability.

The notion that stress erythropoiesis in the spleen may be inherently different from homeostatic erythropoiesis in the bone marrow has been suggested by the observation that BFU-E colonies can be developed, by mouse splenocytes after phenylhydrazine-induced acute hemolytic anemia, in colony assays in medium containing only erythropoietin.[12] Spleen MEP, in contrast to bone marrow MEP, were noted to respond to differentiation stimuli induced by bone morphogenetic protein-4 by creating *stress BFU-E* colonies, under the influence of only a high concentration of erythropoietin.[12] Although mice with mutated cKit (the receptor for stem cell factor) have delayed expansion of stress BFU-E colonies, the final number of such colonies developed is 2-2.5 times greater than that seen in control mice.[13]

Rac GTPases (Rac1, Rac2, and Rac3), a subfamily of the Ras-related Rho GTPases, act as molecular switches in many signaling pathways,[14] and are major regulators of cytoskeletal changes controlling cell shape, adhesion, and migration. Rac1 and Rac3 are ubiquitously expressed, whereas Rac2 is specific to cells of hematopoietic lineage.[14-16] Using conditional gene-targeted mice, we previously demonstrated that combined Rac1 and Rac2 deficiency causes anemia with reticulocytosis, decreased red blood cell deformability, and altered erythrocyte cytoskeleton organization, indicating a specific role for these signaling molecules in structural aspects of erythrocyte biology.[17] Rac1 and Rac2 GTPases have been shown to have both overlapping and distinct roles in cell survival, proliferation, and differentiation in various hematopoietic cell lineages.[15,18] A possible role for Rac GTPases in erythropoiesis has been suggested by the fact that the cKit, the receptor for stem cell factor, activates Vav,[19] a guanine nucleotide exchange factor for Rac1.[20] There is marked transcription of Vav in early stages of erythroid development and this transcription is down-regulated upon initiation of differentiation to erythroblasts,[21] concurrently with the reduction of effects of stem cell factor in erythroid precursors and the increasing activity of erythropoietin in mediating terminal differentiation.[2,22]

In this study, we examined the role of Rac1 and Rac2 GTPases in erythropoiesis in the bone marrow and spleen, investigating whether steady-state and stress erythropoiesis are regulated through different signaling mechanisms.

Design and Methods

Mice

Mx1Cre$^{Tg/+}$;Rac1$^{flox/flox}$;Rac2$^{-/-}$ mice bred on a 129Sv and C57BL/6J background were generated as described previously.[15,17] In order to delete the *Rac1* sequence *in vivo* from hematopoietic cells, including erythroid progenitors and precursors, Cre-mediated recombination was carried out by polyinosinic-polycytidylic acid (pI-pC) (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) treatment of transgenic and wild-type mice using four intraperitoneal injections of pI-pC at a dose of 10 μg/g of weight, administered every other day. Wild-type mice of the same background were subjected to the same treatment to be used as controls. The mice were sacrificed 7-10 days after completion of induction, at which time Rac1 in the bone marrow or spleen cells was decreased by at least 80%, as determined by immunoblotting (*data not shown*). At this time we were able to avoid the increased mortality of Rac1$^{-/-}$;Rac2$^{-/-}$ mice which peaks 2-3 weeks after induction, correlating with the nadir of anemia. All animal protocols were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

Isolation of splenocytes and bone marrow cells

Spleens were gently homogenized and splenocytes were separated from parenchyma by filtration through a 40 μm cell-strainer (BD Biosciences, San Jose, CA, USA). Bone marrow cells were recovered from freshly isolated pelvic bones, femora, and tibiae by

flushing the marrow with Hank's balanced salt solution using a 25-gauge needle and passing through a 40 μm cell-strainer. To calculate total nucleated cells in bone marrow obtained from bilateral pelvic bones, femora, and tibiae or in the spleen, cell counts in concentrated solutions were determined using an automated hematology analyzer (Hemavet 850, Drew Scientific, Oxford, CT, USA).

Histology

Bones and spleens were placed in 10% formalin for fixation overnight. The bones were decalcified in EDTA solution. The fixed tissues were then processed in paraffin and sections (5 μm) were stained with hematoxylin and eosin for routine histological examination.

Colony assays

To identify BFU-E colonies, bone marrow or spleen cells (1×10^5 or 3×10^5 nucleated cells, respectively) were cultured in 1 mL methylcellulose medium, containing 1% methylcellulose (M3134; StemCell Technologies, Inc., Vancouver, CA, USA), 30% fetal bovine serum, 2% penicillin and streptomycin, 1% bovine serum albumin, and 10^{-4} M β-mercaptoethanol, in triplicate in the presence of stem cell factor (100 ng/mL) (PeproTech, Inc., Rocky Hill, NJ, USA), interleukin-3 (10 ng/mL) (PeproTech), granulocyte colony-stimulating factor (100 ng/mL) (Amgen, Inc, Thousand Oaks, CA, USA), and erythropoietin (4 U/mL) (Amgen). For CFU-E assays, bone marrow or spleen (5×10^5 or 1.5×10^6 nucleated cells, respectively) were cultured in 1 mL methylcellulose medium in the presence of stem cell factor (100

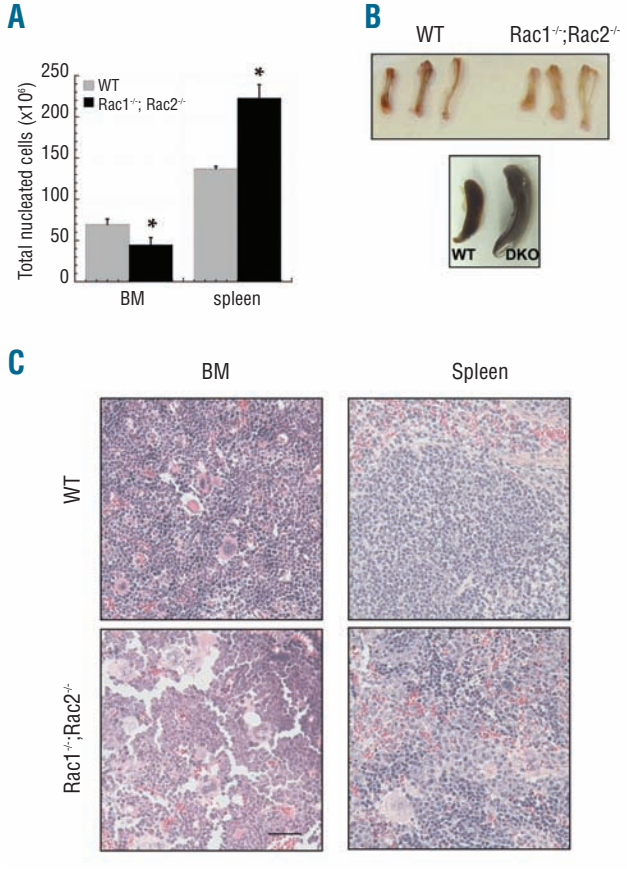

Figure 1. Cellularity and histology of bone marrow (BM) and spleen in Rac1$^{-/-}$;Rac2$^{-/-}$ mice. (A) Total cell count in bone marrow from two pelvic bones + two femora + two tibiae and in spleens from wild-type (WT) and Rac1$^{-/-}$;Rac2$^{-/-}$ mice (n=9 for BM and n=7 for spleen, *P<0.05). (B) Representative gross anatomy of bones (pelvis, femora, and tibiae) and spleens from WT and Rac1$^{-/-}$;Rac2$^{-/-}$ (DKO) mice, showing the typically *pale* bones and splenomegaly of the Rac1$^{-/-}$;Rac2$^{-/-}$ animals. (C) Sections of WT and Rac1$^{-/-}$;Rac2$^{-/-}$ BM and spleen stained with hematoxylin-eosin. Rac1$^{-/-}$;Rac2$^{-/-}$ BM is hypocellular with a decreased number of erythroid precursors (indicated by the paucity of the cells with dark nuclei) while extramedullary erythropoiesis and increased red blood cell sequestration is seen in the Rac1$^{-/-}$; Rac2$^{-/-}$ spleen (bar=50 μm).

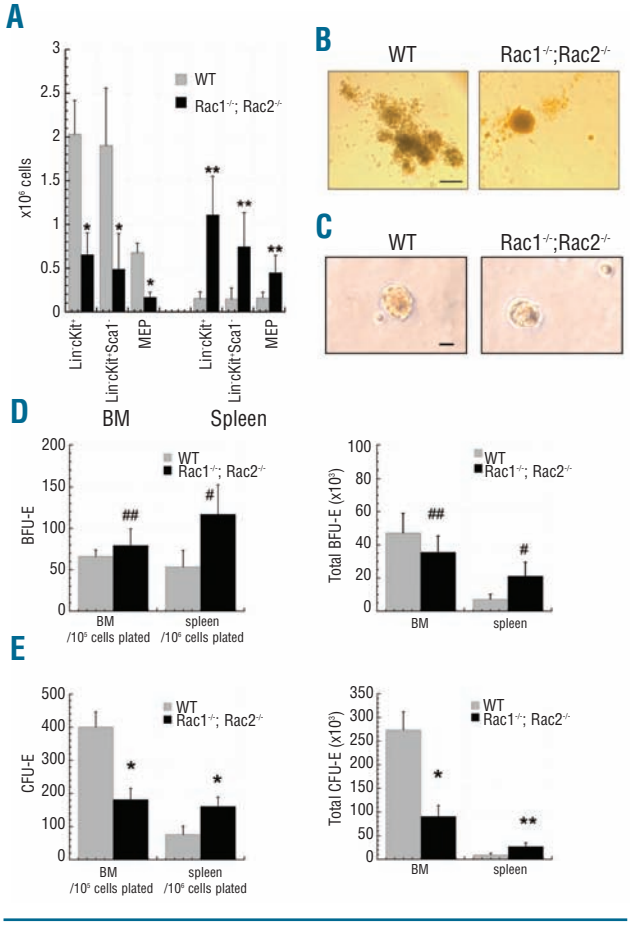

Figure 2. Early hematopoietic and committed erythropoietic progenitors in Rac1$^{-/-}$;Rac2$^{-/-}$ mice. (A) Lin-cKit$^+$, Lin$^-$cKit$^+$Sca1$^-$, and MEP (IL7Rα$^-$Lin$^-$cKit$^+$Sca$^-$FcγR$^-$II/III^{lo}CD34$^-$) cell frequency in bone marrow (BM) and spleen of wild-type (WT) and Rac1$^{-/-}$;Rac2$^{-/-}$ mice (n=3) indicating a decrease of these progenitors in the BM with a compensatory increase in the spleen. (B) BFU-E colonies from Rac1$^{-/-}$; Rac2$^{-/-}$ BM or spleen cells are small and dense without the typical halo of scattered cell-clusters. (bar 100 μm). (C) The benzidine positive clusters of ~8-32 cells fused together, with unclear borders were counted as CFU-E (bar 10 μm). CFU-E colony morphology is similar for the two genotypes. (D) BFU-E frequency and number were similar in the WT and Rac1$^{-/-}$;Rac2$^{-/-}$ BM and spleen. (E) Significant decrease of Rac1$^{-/-}$;Rac2$^{-/-}$ BM CFU-E frequency and content with a corresponding increase in the spleen. For the colony assays (n=9 for BM and n=7 for spleen), from each genotype, each sample was evaluated in triplicate plates. #P=0.15, ##P=0.5, *P<0.05, **P=0.06, between Rac1$^{-/-}$;Rac2$^{-/-}$ and WT bone marrow or spleen.

ng/mL) and erythropoietin (4 U/mL).[23]

Colonies were enumerated, after benzidine staining, using an inverted microscope, after 8 days of incubation at 37°C for BFU-E, and after 48 h for CFU-E. The total number of BFU-E and CFU-E colonies, shown in Figure 2D and E, was calculated by multiplying the ratio of bone marrow and spleen BFU-E and CFU-E colonies (determined as the mean of triplicate plates per mouse) per 1×10^5 or 1×10^6 nucleated cells plated from bone marrow or spleen cells, respectively, by the total number of nucleated cells in bone marrow from bilateral pelvic bones, femora, and tibiae or spleen of the corresponding wild-type and $Rac1^{-/-}$;$Rac2^{-/-}$ mice. BFU-E colony assays were also performed with wild-type bone marrow cells with the Rac inhibitor NSC23766 added gently on the methylcellulose medium, and allowed to diffuse, at concentrations of 0, 50 μM, and 100 μM, on days 0, 3, and 6. BFU-E colonies were identified and photographed on day 8 after benzidine staining.

Flow cytometry

Six-color hematopoietic progenitor cell analysis to identify MEP^4 was performed as previously described[24] using the antibodies: Lin-APC-Cy7, Sca1-PerCP-Cy5.5, IL7Rα-PE-Cy7, CD16/32 (Fcγ Receptor III/II)-PE, c-Kit-APC, and CD34-FITC (BD Biosciences, Franklin Lakes, NJ, USA).

To assess differentiation of erythroblasts in wild-type and $Rac1^{-/-}$;$Rac2^{-/-}$ mice, bone marrow and spleen cells of pI-pC treated animals were immunostained 7-10 days after the final pI-pC injection using fluorescent label-conjugated anti-CD71 and anti-Ter119 (BD Biosciences), as previously described.[10] Concurrent staining for annexin-V and 7-amino-actinomycin D was used to evaluate apoptosis of the different populations (Apoptosis Detection Kit, BD Biosciences).

For assessment of proliferative status of erythroblasts *in vivo*, mice received a single intraperitoneal injection of 0.5 mg BrdU (BD Biosciences). The mice were sacrificed 45 min later, and bone marrow and spleen cells were collected and stained for CD71 and Ter119, then fixed, and stained with APC-conjugated anti-BrdU antibody using the APC-BrdU Flow Kit (BD Biosciences) according to the manufacturer's instructions. Flow-activated cell sorting (FACS) analysis was performed using a FACS Canto Flow Cytometer (Becton Dickinson, Lincoln Park, NJ, USA) and flow cytometry sorting was performed using Facs Aria II (Becton Dickinson) with FacsDiva software v6.1.

Proliferation and apoptosis of the MEP population was evaluated by flow cytometry. Bone marrow and spleen cells were collected 45 min after a single intraperitoneal injection of 0.5 mg BrdU. Since we would have been unable to evaluate staining by more than six colors by flow cytometry, after red blood cell lysis the cells were incubated with biotin-conjugated antibodies for lineage markers (BD Biosciences) and for IL7Rα (Ebioscience, Inc., San Diego, CA, USA) which were then bound to streptavidin-conjugated beads and passed through an autoMACS™ Separator (Miltenyi Biotec Inc., Auburn, CA, USA) that provided bone marrow and spleen progenitors depleted for lineage and IL7Rα markers. This Lin^-;$IL7R\alpha^-$ population was then stained with Sca1-biotin and streptavidin-APC-Cy7(BD Biosciences), CD16/32 (Fcγ Receptor III/II)-PE (BD Biosciences), c-Kit-PE-Cy7 (Ebioscience), and CD34-FITC (BD Biosciences), and either with APC-conjugated anti-BrdU antibody and 7-amino-actinomycin D using the APC-BrdU Flow Kit (BD Biosciences) to determine proliferation, or with APC-conjugated annexin-V and 7-amino-actinomycin D to evaluate apoptosis.

Cytospins were prepared at 500 rpm x 5 min in a Cytospin® 4 Cytocentrifuge (Thermo Shandon Inc, Pittsburgh, PA, USA) and stained with Wright's stain (Harleco EMD).

Measurement of the active (GTP-bound) Rac proteins

The levels of active Rac isoforms in mature erythrocytes were examined by effector domain GST-PAK1 pull-down assay. Briefly, the pull-down assays were performed by using the p21-binding domain of p21-activated kinase 1 fused with glutathione S-transferase bound to glutathione-agarose (Sigma), as previously described.[15,17,25] Total Rac and GTP-bound Rac1 and Rac2 were then visualized by immunoblotting using anti-Rac1,2,3 (Signal Transduction Labs), anti-Rac1 (BD Transduction Laboratories, San Jose, CA, USA), and anti-Rac2 (Novus Biologicals, Littleton, CO, USA) antibody, respectively. Rabbit polyclonal glyceraldehyde phosphate dehydrogenase (GAPDH) antibody (Abcam, Cambridge, MA, USA) was used as a loading control. The antibodies were used at a 1:1000 dilution for immunoblotting.

Statistics

Results are presented as mean ± standard error of mean and are considered significantly different for p values less than 0.05; statistical significance was evaluated using the unpaired Student's t test.

Results

Combined deficiency of Rac1 and Rac2 impairs erythropoiesis in the BFU-E and CFU-E stages of development in the bone marrow but not in the spleen

Erythropoiesis in $Rac1^{-/-}$ or $Rac2^{-/-}$ mice progresses normally,[15,18] pointing to a likely redundancy of Rac GTPase-signaling in erythropoiesis. To determine the effect of combined Rac1 and Rac2 deficiency in the different stages of erythropoiesis, we initially evaluated erythropoietic progenitors in $Rac1^{-/-}$;$Rac2^{-/-}$ mice by flow cytometry and colony assays. In these mice with combined deficiency, total bone marrow cellularity was significantly decreased with a compensatory increase in spleen cellularity (Figure 1A and B). Histological evaluation of wild-type and $Rac1^{-/-}$;$Rac2^{-/-}$ bone marrow and spleen demonstrated decreased cellularity and a paucity of erythroid cells in $Rac1^{-/-}$;$Rac2^{-/-}$ bone marrow along with extramedullary erythropoiesis and increased sequestration in the $Rac1^{-/-}$;$Rac2^{-/-}$ spleen (Figure 1C).

We immunophenotypically analyzed the percentage and absolute numbers of $IL7R\alpha^-Lin^-cKit^+$ cells (which contain almost exclusively the myeloerythroid CFU activity), $IL7R\alpha^-Lin^-cKit^+Sca1^-$ cells (excluding the $Sca1^+$ population which is highly enriched for hematopoietic stem cells), and $IL7R\alpha^-Lin^-cKit^+Sca1^-FcγR\text{-}II/III^{lo}CD34^-$ cells which correspond mainly to the MEP in bone marrow and spleen. Significant redistribution of these early immunophenotypically-defined hematopoietic progenitors was noted, with a decrease of these populations in the $Rac1^{-/-}$;$Rac2^{-/-}$ bone marrow, accompanied by a notable increase in the spleen, in agreement with findings from previous studies of hematopoietic stem cells and progenitors in these mice.[15,18] We demonstrate here that this altered organ location of hematopoietic stem and progenitors cells extends to the first committed erythropoietic progenitors (Figure 2A). The overall sum of the BFU-E and CFU-E activities, as

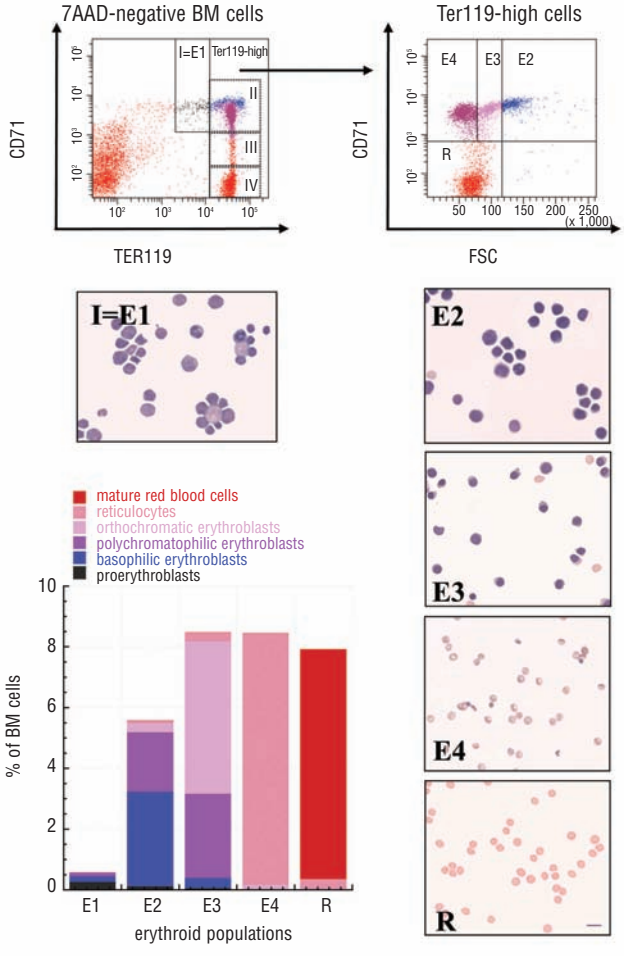

Figure 3. Erythroblast subsets based on CD71 versus FSC sorting. Freshly isolated bone marrow cells were labeled with anti-CD71 and anti-Ter119 antibodies. Dead cells were excluded by staining with 7-amino actinomycin (7-AAD). The Ter119^{high} population from the left panel was further analyzed with respect to intensity of staining for CD71 and size (forward scatter, FSC) in the right panel, as previously shown by Liu *et al.*[26] Three populations (E2, E3, and E4) were discernible in the CD71^{+} area of the right panel in our conditions. Populations I=E1 from the left panel and populations E2, E3, E4, and R from the right panel were sorted and cytospins were stained with Wright's stain (scale bar 10 μm). The differential of 500 cells was obtained in each cytospin from two different sorting experiments and the average is presented in the bar-graph.

determined by colony assays, was consistent with, although not equivalent to, the MEP population content assessed by flow cytometry, likely due to the presence of megakaryocyte-committed progenitors within the MEP population.

The colony assays did not demonstrate a statistically significant change in BFU-E content in bone marrow or spleen of Rac1$^{-/-}$;Rac2$^{-/-}$ animals (Figure 2D), but there was a remarkable difference in the colony morphology. BFU-E colonies from Rac1$^{-/-}$;Rac2$^{-/-}$ mice were small and dense with solid edges and did not have the typical halo of scattered cell-clusters (Figure 2B). This appearance resembled changes previously observed in Rac1$^{-/-}$;Rac2$^{-/-}$ myeloid colonies,[15] suggesting a probable impairment in migration and/or proliferation. BFU-E colonies of wild-type bone marrow cells incubated with the Rac inhibitor NSC23766 demonstrated similar morphological changes (*data not shown*). The CFU-E colony morphology of the two genotypes was similar. However, the CFU-E content was decreased by more than 50% in Rac1$^{-/-}$;Rac2$^{-/-}$ bone marrow while it was increased in the spleen (Figure 2E).

These results indicate that Rac1 and Rac2 have a redundant but essential role in the early erythroid progenitor stages of erythropoiesis in the bone marrow. However, progenitors at the same stages of development in spleen appear to circumvent the deficiency of Rac1 and Rac2 GTPases and demonstrate a compensatory increase.

Combined Rac1 and Rac2 deficiency affects erythroblast populations without impairing cell survival or proliferation

In order to assess erythroid cell differentiation beyond the BFU-E and CFU-E stages, bone marrow and spleen cells of wild-type and Rac1$^{-/-}$;Rac2$^{-/-}$ mice were immunostained for CD71 and Ter119[10] (Figures 3 and 4A and B). The Ter119^{high} cells were further analyzed with respect to their size (using forward scatter), as previously described by Liu *et al.*[26] We observed that under our conditions of staining, using excess of anti-CD71 antibody with the purpose of saturating the CD71 receptor and generating reproducible results, the population II became the major population of the Ter119^{+} cells. This pattern has been demonstrated by several other researchers,[9,27-29] unlike the CD71-Ter119 pattern reported by Socolovsky *et al.*[10,26] in which population IV was the major population. We, therefore, proceeded to flow-sorting and morphological identification of the cells in the E1, E2, E3, E4, and R populations as shown in Figure 3. In agreement with a number of the previous studies in which transferrin receptors were quantified during erythroid development,[8,9,30,31] as well as with a recent report by McGrath *et al.*,[11] the intensity of staining of basophilic, polychromatophilic and orthochromatic erythroblasts with anti-CD71 is similar, ranging within approximately one logarithmic scale and, therefore, these erythroblasts overlap within population II of the CD71-Ter119 cascade. The CD71^{-}Ter119^{high} cells (population IV in the CD71 *versus* Ter119 flow cytogram equivalent to population R in the CD71 *versus* forward scatter plot) are more than 95% mature red blood cells. Based on this immunophenotypic analysis of erythroid precursors, the erythroblast content was found to be significantly decreased in Rac1$^{-/-}$;Rac2$^{-/-}$ bone marrow compared to that in wild-type bone marrow, while it was increased in the spleen (Figure 4C and D). In summary, erythroid progenitors and precursors were severely decreased in the bone marrow, while erythropoiesis in the spleen was increased, mounting an appropriate response to the concurrent hemolytic anemia due to the cytoskeletal defect that we previously described.[17]

To evaluate whether altered apoptosis in the erythroblast stages might play a role in the observed differences of the erythroid populations, we stained the cells with annexin-V and 7-amino-actinomycin D in addition to CD71 and Ter119. No differences were observed in apoptosis between wild-type and Rac1$^{-/-}$;Rac2$^{-/-}$ erythroblasts in bone marrow or spleen (Figure 4C1 and D1), indicating

that apoptosis at these stages of erythropoiesis did not contribute significantly to the altered erythropoiesis in Rac-deficient mice. Additionally, *in vivo* BrdU labeling of wild-type and Rac1$^{-/-}$;Rac2$^{-/-}$ erythroblasts demonstrated no difference in the proliferation of erythroblast populations I and II in bone marrow or spleen (Figure 4C2 and D2).

Combined Rac1 and Rac2 deficiency compromises proliferation of the MEP population in the bone marrow while it allows increased survival and proliferation of the MEP population in the spleen

We proceeded to evaluate apoptosis and proliferation in the stages of erythroid differentiation preceding the erythroblasts, i.e. the erythroid progenitor stages defined by colony assays as the cells having BFU-E and CFU-E activity, and by flow cytometry as a subset of the MEP population. Bone marrow and spleen cells were depleted for lineage and IL7Rα markers and were then stained for Sca-1, c-Kit, CD34, and FcγR III/II, with the aim of gating on the MEP population (IL7Rα$^-$Lin$^-$cKit$^+$Sca1$^-$FcγR-II/III^{lo}CD34$^-$). Using annexin V and 7-amino-actinomycin D staining, Rac1$^{-/-}$;Rac2$^{-/-}$ MEP cells were shown to have similar apoptosis to that of wild-type cells in the bone marrow but 60% decreased apoptosis in the spleen. Using *in vivo* BrdU labeling, proliferation of the Rac1$^{-/-}$;Rac$^{-/-}$ MEP population was shown to be decreased in the bone marrow by approximately 40%, while it was increased two-fold in the spleen in comparison to that in the wild-type population (Figure 5).

These data demonstrate that Rac1 and Rac2 deficiency affects the erythroid differentiation program in the bone marrow in the erythroid progenitor stages, likely through disruption of cytokine-mediated signaling.[2,22,32] This defect of the erythroid progenitors in the bone marrow continues through the erythroblast stages. In contrast, Rac1$^{-/-}$;Rac2$^{-/-}$

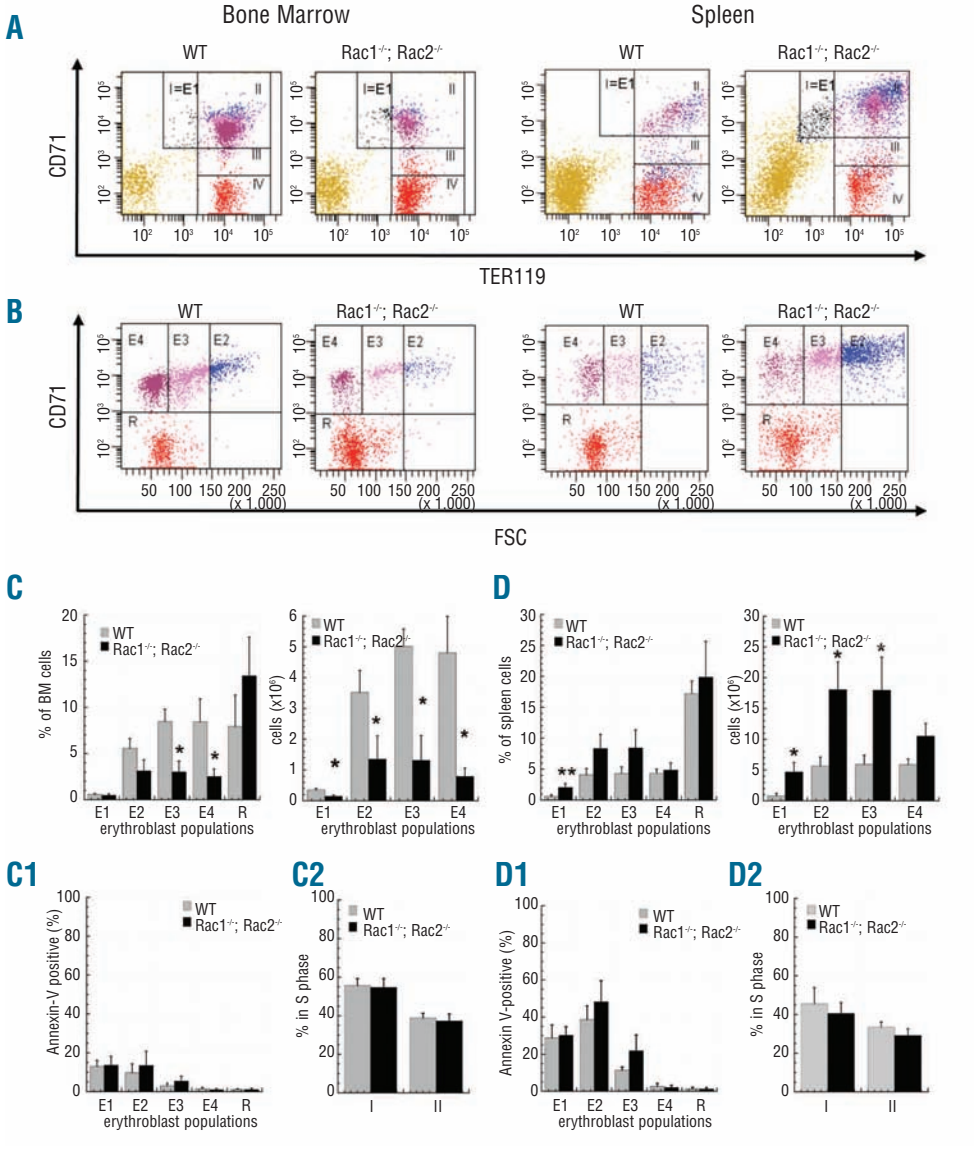

Figure 4. Erythroblast populations in the bone marrow and spleen of Rac1$^{-/-}$; Rac2$^{-/-}$ mice. (A) Representative flow cytograms of erythroblasts stained for CD71 and Ter119 (populations I-IV) from bone marrow (n=6) and spleen (n=4) of wild-type (WT) and Rac1$^{-/-}$;Rac2$^{-/-}$ mice. (B) The corresponding flow cytometric analysis of Ter119^{high} cells in respect to CD71 and size (forward scatter, FSC) for bone marrow and spleen. The frequency of the erythroblast populations E1, E2, E3, E4, and R and the cell count of E1, E2, E3, and E4, as calculated from the percentage of each population multiplying by the corresponding nucleated cell count of (C) bone marrow (mean±SE from six different mice of each genotype) or (D) spleen (mean±SE from four different mice of each genotype). Percentage of annexin-V positive cells out of viable (7-amino actinomycin negative) cells from the erythroblast populations E1, E2, E3, E4 from (C1) bone marrow (mean±SE from six different mice of each genotype) or (D1) spleen (mean±SE from four different mice of each genotype). Percentage of erythroblast populations I and II in S-phase from (C2) bone marrow or (D2) spleen (mean±SE from nine different mice of each genotype). *P<0.05, **P=0.06 between Rac1$^{-/-}$; Rac2$^{-/-}$ and WT bone marrow or spleen.

spleen erythroid progenitors are increased leading to an increased number of erythroid precursors (erythroblasts), which compensate for the defect in erythropoiesis in the bone marrow and for the chronic hemolytic anemia associated with a cytoskeletal defect.[17]

Discussion

We previously demonstrated that Rac1 and Rac2 have a specific role in the regulation of the actin cytoskeleton in structural aspects of red blood cell biology.[17] Here we describe an additional non-structural role for Rac GTPases in regulating erythropoiesis. While splenic erythropoiesis in Rac1$^{-/-}$;Rac2$^{-/-}$ mice is increased, with the animals appropriately mounting reticulocytosis in response to the chronic hemolytic anemia due to the cytoskeletal defect,[17] the erythroid precursors and progenitors in the bone marrow are significantly decreased. To investigate the erythroblast populations in the bone marrow and spleen of our experimental model and control mice, we stained the cells for the surface markers Ter119 and CD71, and analyzed the Ter119^{high} cells by size, as previously described.[10,26]

In our experiments, when staining with an excess of CD71 antibody, population II was consistently the major population of the Ter119^{+} cells in wild-type bone marrow. We, therefore, used FACS to sort the populations produced under these conditions. We demonstrated that the correlation of the morphological characteristics of the erythroblasts within regions I-IV defined by flow cytometry for Ter119 and CD71 is rather broad. Basophilic, polychromatophilic, orthochromatic erythroblasts and reticulocytes grossly overlap within population II of the CD71-Ter119 cascade and extend in a range of 20 to 50-fold difference of fluorescent intensity for CD71 (populations E1, E2, and E3 in Figures 3 and 4B), while the CD71-negative population IV consists mainly of mature red blood cells (Figure 3).

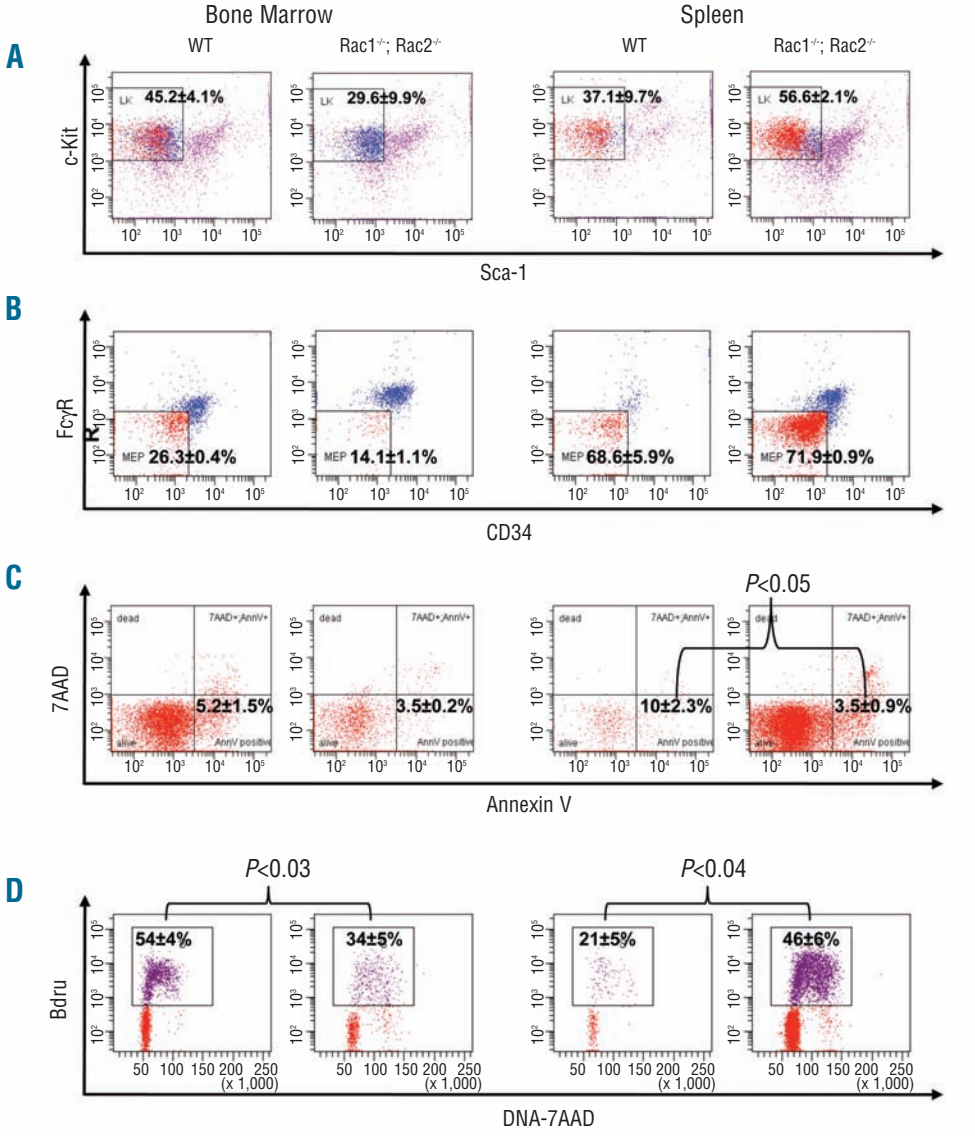

Figure 5. The Rac1$^{-/-}$;Rac2$^{-/-}$ MEP population demonstrates decreased proliferation in the bone marrow (BM) while it has increased proliferation and decreased apoptosis in the spleen. BM and spleen cells from wild-type (WT) and Rac1$^{-/-}$;Rac2$^{-/-}$ mice (n=3), depleted for lineage and IL7Rα markers, were stained for Sca-1, c-Kit, CD34, and FcγR III/II. (A) The Lin^{-}IL7Rα^{-}cKit^{+}Sca1^{-} cells are depicted as the LK population (percentage shown out of Lin^{-}IL7Rα^{-} cells) further analyzed in (B) where the MEP population is shown with the flow cytometric phenotype of Lin^{-}IL7Rα^{-}cKit^{+}Sca1-FcγR-II/III^{lo}CD34^{-} (percentage shown out of LK cells). (C) Staining with annexin-V and 7-amino actinomycin (7AAD) indicates that the Rac1$^{-/-}$;Rac2$^{-/-}$ MEP cells have similar apoptosis as the WT cells in the BM but significantly decreased apoptosis in the spleen. (D) BM and spleen cells labeled with BrdU by an *in vivo* intraperitoneal injection 45 min prior to euthanasia and isolation of the corresponding tissue, demonstrate that proliferation of the Rac1$^{-/-}$;Rac2$^{-/-}$ MEP population is significantly decreased in the BM but increased in the spleen.

The number of transferrin receptors in the erythroblast populations was quantified early on by measuring binding of ^{125}I-labeled transferrin. A decline in the number of transferrin receptors, which varied from 8- to 23-fold, was shown from the least mature erythroblasts to reticulocytes.[8,30,31] By flow cytometry, after staining the transferrin receptors with fluorescently labeled anti-CD71, Loken *et al.* demonstrated that the fluorescence intensity for the transferrin receptor on basophilic and orthochromatic erythroblasts ranged within an approximately 1.5 increment of the logarithmic scale (a 50-fold difference),[9] while Socolovsky *et al.* found the range to be in the order of magnitude of 10^3 (1,000-fold difference).[10,26] Using a new flow cytometric technology that enables parallel evaluation of morphology (ImageStream, Amnis Corporation, Seattle, WA, USA), McGrath *et al.* showed that the difference in intensity of staining with anti-CD71 ranged from 100,000 to 2,000 (a 50-fold difference).[11]

The distribution of proerythroblasts, and basophilic, polychromatophilic, and orthochromatic erythroblasts, as shown in the bar graph of Figure 3, is in agreement with the observation that the number of cells in each sequential stage of erythroblasts gradually increases as they differentiate. Furthermore, this distribution is concordant with published data on the relative amounts of transferrin receptor during the progression of erythropoiesis, which have repeatedly shown that erythroid precursors up to the reticulocyte stage retain CD71; the cells then lose the receptor completely and become a truly CD71-negative population when they fully mature to erythrocytes. This re-evaluation of erythroblast distribution by flow cytometry characteristics, based on Ter119 and CD71 staining, allows an accurate interpretation of our results on erythroblast differentiation in the bone marrow and spleen of Rac1$^{-/-}$;Rac2$^{-/-}$ mice. As shown in Figure 4, the decrease in the erythroblast population in the Rac1$^{-/-}$;Rac2$^{-/-}$ bone marrow and the increase in the spleen can be tracked back to the least mature population indicating that the bone marrow defect occurs in stages of erythropoiesis preceding that of erythroblast generation, i.e. the BFU-E and CFU-E stages as defined by colony assays, or the MEP population, as defined by flow cytometry.

This regular erythroblast progression in Rac1$^{-/-}$;Rac2$^{-/-}$ bone marrow and spleen is compatible with the lack of difference in either apoptosis or proliferation that we observed between wild-type and Rac1$^{-/-}$;Rac2$^{-/-}$ erythroblast populations. The change in bone marrow erythropoiesis elicited by Rac1 and Rac2 deficiency appears to happen within the MEP population. Colony assays confirm this early defect, demonstrating abnormal BFU-E colony morphology and a decreased number of bone marrow CFU-E.

In contrast, splenic erythropoiesis is increased. We developed a protocol to study *in vivo* proliferation and apoptosis of the MEP population, and found that combined Rac1 and Rac2 deficiency compromises proliferation of the bone marrow MEP population, while it allows increased survival and proliferation of spleen MEP, which are able to create an increased number of erythroblasts in the spleen, mounting successful stress erythropoiesis.

The erythroid progenitors are compromised by Rac1 and Rac2 deficiency in the bone marrow, but not in the splenic microenvironment. A similar microenvironmental preference was previously noted for Rac1$^{-/-}$;Rac2$^{-/-}$ hematopoietic stem and progenitor cells.[18] Rac deficiency may disrupt signaling downstream of the early erythropoietic cytokine stem cell factor and, possibly interleukin-3, to affect bone marrow erythropoiesis. However, stress erythropoiesis in the spleen can proceed in the absence of Rac1 and Rac2, possibly due to a unique erythropoietic inductive microenvironment,[33] in which cytokines that act preferentially in the spleen, such as bone morphogenetic protein-4, exert their function,[12,13] or due to a different contribution of erythropoietic cytokines towards extramedullary erythropoiesis. Stem cell factor and erythropoietin play major roles in stress erythropoiesis.[34,35] Although Rac1 has been shown downstream of the stem cell factor-c-kit signaling axis,[19,20] the role of Rac GTPases in erythroid progenitors in response to interleukin-3 signaling needs to be investigated further, since interleukin-3 is an erythropoietic cytokine that may play a more significant role in the early stages of steady-state, medullary erythropoiesis rather than in extramedullary, stress erythropoiesis.

The 'stress BFU-E' previously described as resident in the spleen has been shown to require only erythropoietin at relatively high levels to develop and this pathway may circumvent Rac1 and Rac2 deficiency.[12] Phenylhydrazine-induced acute hemolysis in Rac1$^{-/-}$;Rac2$^{-/-}$ mice led to a reduction in CFU-E in the bone marrow and an additional increase of CFU-E in the spleen (*data not shown*). It is, therefore, likely that in our animal model, the erythroid progenitors that support stress erythropoiesis are not exclusively endogenous to the spleen and there may be migration of Rac1$^{-/-}$;Rac2$^{-/-}$ early erythroid progenitors from the bone marrow to spleen, where they thrive and support erythropoiesis effectively.[36,37]

In summary, in addition to the defects in mature erythrocyte cytoskeleton previously demonstrated in Rac1$^{-/-}$;Rac2$^{-/-}$ mice,[17] we demonstrate here that deficiency of Rac1 and Rac2 GTPases causes defects in erythropoiesis specifically in the bone marrow, with these defects possibly being mediated by an interruption of intracellular signal transduction of early erythropoietic cytokines, such as stem cell factor and interleukin-3. In contrast, stress erythropoiesis in the splenic microenvironment and/or under the influence of erythropoietin or cytokines with preferential action in the spleen can circumvent the deficiency of Rac GTPases successfully.

Further studies of the Rac-regulated molecular pathways in erythroid cells will help in the elucidation of the signaling pathways controlling red cell development and the differences between steady-state and stress erythropoiesis.

Authorship and Disclosures

TAK designed and performed research, analyzed data, and wrote the paper; SP, XZ, JFJ, and DD performed research and analyzed data; DP and HG designed research and analyzed data; JAC, DAW, and YZ contributed instrumental suggestions and mentorship on the research design, analysis of data, and writing of the paper.

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