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Rho GTPases in Erythroid Maturation

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Abstract

Purpose of review—The present review summarizes our current understanding of the roles of Rho GTPases in early erythropoiesis, downstream of cytokine signaling, and in terminal erythroblast maturation and enucleation, as master regulators of the cytoskeleton and cytokinesis.

Recent findings—Similarities of structural and signaling requirements of erythroblast enucleation with the cytokinesis process have been confirmed and expanded in the last year, suggesting that enucleation is a form of asymmetric cell division. Myosin, the classic actin partner in cytokinesis, was shown to play an essential role in enucleation. Studies with multispectral high-speed cell imaging in flow demonstrated a sequential process requiring establishment of polarity through a unipolar microtubule spindle in orthochromatic erythroblasts, followed by Rac-directed formation of contractile actomyosin ring and coalescence of lipid rafts between reticulocyte and pyrenocyte, steps which reiterate the choreography of cytokinesis. mDia2, a Rho-effector known to play a role in enucleation, was also found essential for erythroblast cytokinesis since its deficiency in mice caused failure of primitive erythropoiesis and embryonic death.

Summary—Further elucidation of the role of Rho GTPases in the erythroid lineage development may reveal potential targets for improving red blood cell production *in vivo* and *in vitro*.

Keywords

Erythropoiesis; erythroblast enucleation; Rho GTPases; RhoA; Rac; Cdc42

INTRODUCTION

Rho (**Ras homology**) GTPases belong to the Ras superfamily of small G proteins. They are found in all eukaryotic organisms and regulate a wide spectrum of cellular functions including actin cytoskeleton, cell polarity and motility, microtubule dynamics, and vesicular transport pathways, as well as survival and cell cycle [1–5]. The classical Rho GTPases, comprising the subfamilies Rho (RhoA, RhoB, and RhoC), Rac (Rac1, Rac2, Rac3, and RhoG), Cdc42 (Cdc42, TC10 or RhoQ, and TCL or RhoJ), RhoF and RhoD, switch between the inactive GDP-bound form and the active GTP-bound form, in response to a variety of cell signals. Upon activation, they interact with multiple downstream effector proteins in a

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spatially and temporally controlled manner [6]. Rho GTPases are typically modified post-translationally by lipid moieties so they can localize to specific cell membranes where they modulate membrane dynamics and mediate signaling [7]. For example, palmitoylation of Rac1 at the C-terminus, following its geranylgeranylation, leads to its translocation to the cholesterol-rich, detergent-resistant subdomains in the plasma membrane known as lipid rafts, upon stimulation by cytokines or adhesion interactions [8].

Initial studies on the role of Rho GTPases in hematopoiesis utilized overexpression of constitutively active or dominant-negative mutants, which suffer from limited specificity, variable dosage, and uncontrolled spatial and temporal distribution in cells. Recent gene targeting of individual Rho GTPases in mice has allowed studies of their role in primary cells under more physiological conditions [9]. Pharmacological inhibitors are also available in assisting the study of the role of Rho GTPases during a specific stage of a hematopoietic lineage progression [10,11]. A network of cross-talking and collaborating Rho GTPases are found involved in mediating complex signaling events in the dynamic settings of hematopoiesis [12].

ERYTHROID COMMITMENT AND EARLY ERYTHROPOIESIS

In the erythroid lineage, the earliest committed progenitors in the mouse bone marrow are the megakaryocyte-erythroid progenitors (MEPs). This population consists of cells with burst forming unit-erythroid (BFU-E), colony forming unit-erythroid (CFU-E) and CFU-megakaryocytic (CFU-Meg) potential [13,14]. The process of erythropoiesis is under tight regulation by cytokines including stem cell factor (SCF), interleukin-3 (IL3), and erythropoietin (Epo), other growth factors including steroids, and the microenvironment, which may vary depending on the developmental stage and the RBC production needs [15–17]. Rho GTPases such as Rac (Rac1, Rac2, and Rac3), Cdc42, and RhoA have been implicated in signaling at the erythroid progenitor commitment and proliferation stages, downstream of cytokines and upstream of transcription factor network.

Rac GTPases are essential for normal erythropoiesis in the bone marrow but dispensable for stress erythropoiesis in the spleen

Among the three Rac GTPases, Rac1 and Rac3 are ubiquitously expressed while Rac2 is expressed only in hematopoietic cells while Rac2 is expressed only in hematopoietic cells [18–20]. Rac1 and Rac2 GTPases were the first Rho GTPases shown to play vital distinct as well as overlapping roles in hematopoietic stem cells/progenitors (HSC/Ps) and mature blood cells. Both Rac isoforms regulate homing, engraftment, actin cytoskeleton, cell survival, and proliferation of HSC/Ps [21,22]. In neutrophils, Rac1 controls cell spreading and adhesion, while Rac2 regulates directed migration and superoxide production [21,23].

Rac1 is indispensable for embryonic development [24], therefore combined deficiency of Rac1 and Rac2 in hematopoietic cells can be attained in gene-targeted mice with germline deletion of *Rac2* gene and conditional *Rac1^{flox/flox}* alleles with a *Cre* transgene driven by the *Mx1* promoter. Cre recombinase is expressed in the hematopoietic cells after intraperitoneal injections of polyinosinic-polycytidylic acid (pI-pC) leading to *Rac1* deletion and the development of *Mx1-Cre^{Tg/+};Rac1^{-/-};Rac2^{-/-}* hematopoietic cells, including Rac1 and

Rac2-deficient erythroid progenitors and precursors [25,26]. Deletion of either *Rac1* or *Rac2* in hematopoietic lineages led to no significant abnormalities in erythropoiesis or the mature red blood cells (RBCs), suggesting a redundancy for these two GTPases in the erythroid lineage.

The number of cells with BFU-E activity in *Rac1*^{+/−};*Rac2*^{−/−} mouse bone marrow is slightly decreased but most notably affected is the morphology of the Rac-deficient colonies which are small, round, and condensed. In erythroid differentiation sequence, CFU-E and erythroblasts are significantly decreased in *Rac1*^{+/−};*Rac2*^{−/−} bone marrow. MEPs of the bone marrow (including BFU-E, CFU-E, and CFU-Meg) are significantly reduced with decreased proliferation whereas, in contrast, they are significantly increased in the spleen, particularly the subset population of CFU-E mounting stress erythropoiesis [26]. Similarly with the *Rac1* and *Rac2*-deficient HSC/Ps [21], MEPs demonstrate increased homing in the spleen as well as increased proliferation and decreased apoptosis [26]. *Rac1* and *Rac2* GTPases have been shown in HSC/Ps to be downstream of the c-kit receptor, promoting proliferation and survival, upon SCF stimulation [21]. Similarly, *Rac1/Rac2* deficiency compromises baseline erythropoiesis in the early stages mediated by SCF (Figure 1A). However, within the splenic microenvironment or under the influence of unique cytokines acting in the spleen during stress erythropoiesis, like bone morphogenetic protein 4 (BMP4) [27], *Rac1/Rac2* appear to be dispensable for erythroid progenitor proliferation and differentiation [28].

Cdc42 regulates the balance between myelopoiesis and erythropoiesis

The role of Cdc42 in erythropoiesis has been studied in mice with either deletion of a negative regulator, *Cdc42GAP* or an inducible deletion of *Cdc42* in hematopoietic lineages [29,30]. Homozygous deletion of *Cdc42GAP*, the GTPase-activating protein of Cdc42, which downregulates Cdc42 activity by promoting the hydrolysis of GTP to GDP, results to a 3-fold increase in Cdc42 activity in mouse fetal liver and bone marrow (BM) cells. These mice suffer massive neonatal mortality (up to 93% within 1 week of birth) with the surviving ones displaying significant anemia and decreased fetal liver and BM cellularity, along with a reduction of cells with BFU-E and CFU-E activity, as well as CFU-GEMM (granulocyte, erythroid, macrophage, megakaryocyte potential) but normal CFU-GM [29]. This decrease in both erythroid and myeloid progenitors correlates with increased apoptosis associated with increased JNK phosphorylation and reduction of the survival factor BID [29]. While such increased Cdc42 activity affects erythropoiesis by inducing apoptosis, decreased Cdc42 also negatively impacts on erythropoiesis by altering a key transcription factor program. Deletion of *Cdc42* from HSC/Ps leads to development of a fatal myeloproliferative disorder along with a fast decline of erythropoiesis, caused by a significant decrease in the MEP population, as well as in BFU-E and CFU-E activities, leading to profound anemia. These effects appear to be mediated by downregulation of the pro-erythroid transcription factor GATA2 and upregulation of the pro-myeloid transcription factors PU.1, C/EBPα, and Gfi-1 [30].

RhoA is essential for progenitor commitment and proliferation

The role of RhoA in hematopoiesis is less studied compared with that of Rac1, Rac2, and Cdc42. Hematopoietic specific deletion of *RhoA* in *MxCre^{Tg/+};RhoA^{fllox/fllox}* mice leads to a development of fatal aplastic anemia since *RhoA*^{-/-} HSCs retain long-term engraftment potential but fail to produce multipotent progenitors and lineage-defined blood cells [31], limiting the ability to derive the effect of RhoA in the erythroid lineage in this model. It will be an interesting area to pursue using an erythroid lineage specific genetic mouse model in the future, as RhoA has been shown to be regulated by SCF in human erythroid progenitors [32].

TERMINAL ERYTHROID MATURATION AND ENUCLEATION

CFU-E subpopulation cells give rise to the morphologically recognizable proerythroblasts, which differentiate through four to five successive divisions into orthochromatic erythroblasts that enucleate to produce reticulocytes [33]. The signaling role of Rho GTPases in regulating the terminal erythroid maturation and enucleation is beginning to be appreciated.

Evolving understanding of the role of RhoA in erythroid maturation

After downregulation of the cKIT receptor at the proerythroblast stage, RhoA activity is no further stimulated by SCF [32]. Moreover, the Rho-associated kinase (ROCK-1), a major RhoA effector, was found to be activated at the erythroblast stage in culture by caspase-3-mediated cleavage, independently of Rho signaling, and to promote phosphorylation of the light chain of myosin II [34]. Inhibition of ROCK by the small molecule inhibitor Y27632 or by shRNA causes a decrease of erythroblast maturation *in vitro* [32]. In contrast, deletion of *ROCK1* in mice enhances the response to stress erythropoiesis after phenylhydrazine-induced hemolysis. Constitutional ROCK1 deficiency decreased the level of reactive oxygen species (ROS) production in the bone marrow and spleen erythroid cells, reduced the expression and phosphorylation of p53, and reduced caspase-3 cleavage promoting erythroblast survival, implicating ROCK1 as a negative regulation of stress erythropoiesis.

Mice with constitutive gene deletion of *mDia2*, a formin isoform and Rho effector that typically nucleates unbranched actin filaments, were recently described [35]. This mouse model is embryonic lethal, survives up to embryonic day 11.5 (E11.5) and exhibits severe anemia with multinucleated primitive red blood cells. BFU-E and CFU-E colonies can be produced by mDia2-deficient fetal liver cells at E11.5, although the colony numbers are significantly decreased. Erythroblast cytokinesis fails with increasing rate as the size of the erythroblasts declines along with the progression of erythroid maturation. mDia2-deficient erythroblasts are able to enucleate although with decreased frequency. Cytochalasin-D or NSC23766 [10], inhibiting filamentous (F)-actin polymerization or Rac GTPases respectively, block nucleus protrusion completely when directly applied to an erythroblast under microscopic observation [35]. However, when the same inhibitors are applied to erythropoiesis cultures *in vitro* and the whole population is examined, the block of enucleation is not complete either [36,37]. Downregulation of mDia2 by small interfering RNA (siRNA) in fetal liver cell culture similarly decreases erythroblast enucleation whereas

overexpression of a constitutively active mutant of mDia2 rescues the enucleation defect induced by inhibition of Rac GTPases [36], placing mDia2 downstream of Rac in this process. The conclusion that mDia2 may be more essential for the erythroblast maturation rather than for enucleation, therefore, needs to be cautioned.

The human homolog of mDia1 (*DIAPH1* gene) localizes in human chromosome 5q31.3 and its loss may contribute to the development of myelodysplastic syndrome (MDS) with interstitial deletion of 5q (5q⁻ MDS), which carries high risk for leukemia [38]. Mice with targeted deletion of the murine *DIAPH1* homolog *Drf1* develop splenomegaly, fibrotic and hypercellular bone marrow, and extramedullary hematopoiesis in both spleen and liver including expansion of erythroid precursors [39]. Additional deletion of RhoB, one of the upstream GTPases for mDia1, accelerates MDS development and is associated with increased RBC dysplasia [38].

Rac GTPases promote erythroblast enucleation

Cultured mouse fetal liver erythroblasts infected with retrovirus overexpressing constitutively active or dominant negative Rac1 or Rac2 mutant have significantly decreased enucleation, indicating that excessive activation or inhibition of Rac GTPases *in vitro* inhibits enucleation [36]. Pharmacologic inhibition of Rac GTPases by NSC23766, an inhibitor for all Rac GTPases [10], also inhibits enucleation [36,37,40]. However, enucleation is not compromised in Rac1^{-/-};Rac2^{-/-} erythroblasts, where Rac3 is upregulated and may compensate for Rac1 and Rac2 deficiency [37,40]. Erythroblast enucleation is a process vital for mammalian life; therefore, such redundancy may have been built into the signaling mechanism supporting this process *in vivo*.

Molecules that are involved in cytokinesis were noted to participate in erythroblast enucleation over 25 years ago. F-actin bundles were observed to concentrate between the extruding nucleus and nascent reticulocyte [41], while microtubules (MT) were also found to be essential for the nuclear extrusion process by detailed *in vivo* and *in vitro* studies of rat erythroblasts after treatment with colchicine [42]. Non-muscle myosin IIB was shown recently to play an essential role in erythroblast enucleation, interacting with F-actin at the cleavage furrow between reticulocyte and nucleus and contributing to a contractile actomyosin ring [43]. MT, F-actin, and myosin are classic collaborators in cytokinesis: MT are organized in astral spindles around centrosomes at the poles; during anaphase the pole–pole spacing increases by further MT polymerization and a scaffold organized by RhoA and anillin at the spindle mid-zone determines the position of the division plane [44]. This formation of a highly localized zone of RhoA activity happens before contractile ring formation and even in the absence of actin accumulation [45]. In sequence, detergent-resistant membrane domains (lipid rafts), concentrate in the furrow [46], which ingresses during telophase, developing a barrier between the daughter cells and constricts the spindle mid-zone (the array of interpolar MTs lying between separated chromatids) into a structure called the midbody. During abscission, the furrow “seals” by vesicular trafficking of endosomes, which fuse to create new membranes that separate the daughter cells [47,48].

In agreement with the analogies noted above between erythroblast enucleation and cytokinesis, immunofluorescence staining of enucleating erythroblasts and analysis by

multiparameter high-speed cell imaging in flow revealed a unipolar spindle of microtubules in the polarized orthochromatic erythroblasts preparing for enucleation (Figure 2). F-actin and myosin, labeled at phosphorylated myosin regulatory light chain (pMRLC), assemble into an actomyosin ring between nascent reticulocyte and nucleus in the population of enucleating erythroblasts, and the lipid raft marker GM1 ganglioside concentrates in the furrow [37]. Erythroblasts treated with NSC23766 to inhibit Rac activity, cytochalasin-D or colchicine to inhibit actin or tubulin polymerization respectively, ML7 to inhibit MRLC phosphorylation, or filipin to cause cholesterol depletion and inhibit lipid raft assembly, exhibited decreased enucleation efficiency. As assessed by multiparameter high-speed cell imaging in flow, colchicine inhibited erythroblast polarization implicating microtubules during the preparatory stage of enucleation, while NSC23766 led to absence of lipid raft assembly in the reticulocyte-pyrenocyte border [37]. These results support the notion that enucleation is a form of asymmetric cytokinesis, requiring establishment of cell polarity through microtubule function, followed by formation of a contractile actomyosin ring and coalescence of lipid rafts between reticulocyte and pyrenocyte [49,50].

An accumulation of vacuoles in the cytoplasm proximal to the extruding nucleus has been observed during erythroblast enucleation, indicating that vesicle trafficking contributes to the creation of new membranes necessary for separating reticulocyte from pyrenocyte [51,52]. Chemical inhibitors of vesicle trafficking or knock-down of clathrin that blocks endocytosis inhibit erythroblast enucleation [52], pointing to another similarity with cytokinesis where endosomes fuse to create new membrane that completely separates the daughter cells [47,48]. Vesicle trafficking is typically regulated by Rho GTPases [7]; more work is needed to identify the GTPase(s)-mediated mechanism orchestrating this final event in enucleation.

Signaling of Rho GTPases in mature RBCs

Mice deficient of Rac1 and Rac2 GTPases in their hematopoietic cells exhibit hemolytic anemia due to disturbed actin oligomerization in the erythrocyte cytoskeleton resulting in decreased deformability and increased fragility of the red blood cells under shear stress in the circulation [25]. Adducin, the capping protein for the actin oligomer at the fast growing barbed end, shows increased phosphorylation at Ser724, a protein kinase C (PKC) target, in the Rac1^{-/-};Rac2^{-/-} RBCs; this likely facilitates uncapping, promotion of actin polymerization, and actin dissociation from spectrin [25,53,54]. The ensuing abnormal cytoskeleton may be the result of an abnormal assembly during erythroblast maturation, however one cannot exclude the possibility that there is also dynamic regulation of the actin oligomers by Rac GTPases within the mature RBCs in the circulation.

Rac GTPases contribute to the generation of reactive oxygen species (ROS) in human mature RBCs via NADPH oxidase, downstream intracellular Ca²⁺ and PKC signaling [55]. Rac-GTP is known to participate as a functional subunit of the NOX1 and NOX2 isoforms of NADPH oxidase [56]. This enzymatically mediated production of ROS is higher in the erythrocytes of patients with sickle cell disease (SCD), being stimulated by increased inflammatory cytokines in the plasma, like TGFβ1 and ET-1 via their receptors on the RBC membrane, raising the possibility for targeted therapy in SCD.

CONCLUSION

Rho GTPases play important regulatory roles in all stages of erythropoiesis and are vital for the process of erythroblast enucleation. Recent mouse genetic studies have placed them in a signaling network of primitive and definitive erythropoiesis as well as stress erythropoiesis. Further elucidation of the pathways involving various members of Rho GTPases in erythroid biology may offer insights and therapeutic principles for the management of diseases characterized by defective erythropoiesis and/or hemolysis such as SCD, as well as for advancing the efficiency of red blood cell production in vitro.

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KEY POINTS

- Rho GTPases regulate homing, actin cytoskeleton, cell survival, and proliferation of erythroid progenitors, in response to erythropoietic cytokines like SCF.
- Recent studies demonstrate that erythroblast enucleation is a form of asymmetric cell division, where Rho GTPases and their effectors are employed in roles analogous to the ones they play in cytokinesis.
- Understanding the signaling pathways involving various members of Rho GTPases in erythroid biology may offer insights and therapeutic targets for improving erythropoiesis *in vivo* and *in vitro*.

Figure 1.

Rac1 and Rac2 GTPases mediate homing and proliferation of erythroid progenitors in the bone marrow microenvironment. Their combined deficiency results in massive mobilization of progenitors and increased homing in the spleen. Cdc42 regulates the balance between pro-erythroid and pro-myeloid transcription factors in HSC/Ps. RhoA activity is stimulated by SCF via the c-kit receptor but the ensuing effects in erythroid progenitors have yet to be fully investigated.

Figure 2.

A. Representative images obtained by multispectral high-speed cell imaging in flow show the formation of a unipolar microtubule spindle in orthochromatic WT erythroblasts, and F-actin, phosphorylated myosin regulatory light chain (pMRLC), and lipid rafts at the cleavage furrow between reticulocyte and pyrenocyte in enucleating cells. **B.** A working model for the cytoskeleton and signaling components participating in the erythroblast enucleation process. Microtubules form a unipolar spindle that polarizes the cell and determines the “division plane”. F-actin polymerizes under the control of Rac GTPases and assembles with

myosin to form an actomyosin ring in the cleavage furrow between nucleus and incipient reticulocyte, where lipid rafts coalesce and position active Rac. Endocytic vesicles move and fuse at the division plane to separate the incipient reticulocyte from the nucleus. Rho GTPase-controlled mechanisms (marked by question marks) that may regulate the microtubules, the phosphorylation of MRLC allowing the actomyosin complex to contract, and the movement and fusion of endocytic vesicles are still under investigation.