



# Human erythrocytes: cytoskeleton and its origin

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## Abstract

In the last few years, erythrocytes have emerged as the main determinant of blood rheology. In mammals, these cells are devoid of nuclei and are, therefore, unable to divide. Consequently, all circulating erythrocytes come from erythropoiesis, a process in the bone marrow in which several modifications are induced in the expression of membrane and cytoskeletal proteins, and different vertical and horizontal interactions are established between them. Cytoskeleton components play an important role in this process, which explains why they and the interaction between them have been the focus of much recent research. Moreover, in mature erythrocytes, the cytoskeleton integrity is also essential, because the cytoskeleton confers remarkable deformability and stability on the erythrocytes, thus enabling them to undergo deformation in microcirculation. Defects in the cytoskeleton produce changes in erythrocyte deformability and stability, affecting cell viability and rheological properties. Such abnormalities are seen in different pathologies of special interest, such as different types of anemia, hypertension, and diabetes, among others. This review highlights the main findings in mammalian erythrocytes and their progenitors regarding the presence, conformation and function of the three main components of the cytoskeleton: actin, intermediate filaments, and tubulin.

**Keywords** Erythrocytes · Erythropoiesis · Cytoskeleton · Tubulin · Actin · Intermediate filaments

## Introduction

Erythrocytes, the most quantitatively important components of whole blood, are known to have extensive communication with their extracellular milieu [1]. Moreover, the local dynamics and interactions between these cells are important to determine blood rheology and control the proper perfusion of the tissues [2]. Erythrocytes from mammals are unique among eukaryotic cells because they lack nuclei, cytoplasmic structures and organelles and are therefore unable to synthesize new proteins. Their lack of a nucleus also prevents their proliferation, so circulating mature erythrocytes are the final stage of the development process called

erythropoiesis. Erythropoiesis consists of two phases: proliferation, in which progenitor cells expand and maturation, in which the first morphologically recognizable erythroid cell (the pro-erythroblast) becomes unable to proliferate and undergoes differentiation processes [3, 4]. The culmination of the process is enucleation, where the nucleus is expelled and the resulting enucleated reticulocytes emerge into circulation and rapidly develop into mature erythrocytes [5].

Erythropoiesis involves significant changes in the cells. Given the importance that the plasma membrane and the membrane cytoskeleton have in the functioning of erythrocytes, the composition and interaction between the components of these structures are extensively modified during erythropoiesis. However, these changes are poorly understood, especially in terms of the role played by tubulin and microtubules. This review goes over the main reports on protein changes in the cytoskeleton during erythropoiesis and focuses on the proposed structure and function of tubulin in the erythrocytes, the final product of this process.

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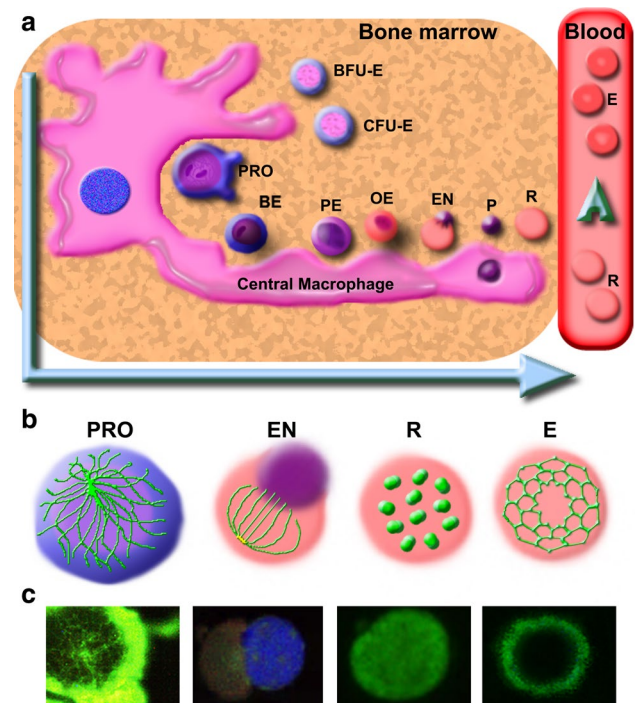
## Erythropoiesis and changes in the skeleton components

Erythrocytes originate from hematopoietic stem cells (HSCs), the most undifferentiated progenitors that become progressively restricted to several or single lineages [6]. Differentiation of HSCs within the erythroid lineage is required for their maturation into red blood cells, and it occurs as part of a balance with proliferation, which is necessary to maintain their renewal capacity [7]. In adult mammals, differentiation occurs in the bone marrow. However, when bone marrow function is insufficient, extramedullary hematopoiesis known as stress erythropoiesis occurs as a physiological compensatory phenomenon [8]. The sites commonly involved in extramedullary hematopoiesis include the liver, spleen, lymph nodes, and most commonly, the paravertebral regions. These sites are thought to be involved in active hematopoiesis during fetal life, and although their activity ceases at birth, the extramedullary hemopoietic vascular connective tissues retain the ability to produce red cells when necessary [9]. In this way, stress erythropoiesis is observed in many hematological conditions (e.g. myelofibrosis, leukemia, lymphoma) but is most common in chronic hemolytic anemias such as thalassemia and sickle cell anemia.

In the bone marrow, erythropoiesis is observed in a complex called erythroblastic island [10, 11]. Erythroblastic islands are composed of several erythroblasts surrounding a central macrophage, and the interactions between erythroblasts and macrophages are essential for normal erythropoiesis [12].

Central macrophages play diverse roles in the process. For example, they provide signals for the normal proliferation of erythroblasts and stabilize cell numbers in the islands. Simulations have shown that islands without macrophages either grow exponentially or disappear after several cell cycles [13]. They are also necessary for an adequate response in stressful situations, such as acute anemia [14], and they synthesize ferritin, which is taken up by erythroblasts for the synthesis of hemoglobin [15]. This provides further evidence for the role of “nurse” macrophages in iron trafficking towards maturing erythroblasts [16]. Central macrophages also promote erythroblast enucleation and phagocytosis of the extruded nuclei, known as pyrenocytes [17].

During erythropoiesis (Fig. 1a), stem cells generate two erythroid progenitor cells. The burst-forming unit-erythroid (BFU-E) is the first cell committed toward the erythroid lineage, and it proliferates and matures into the second progenitor, the colony-forming unit E (CFU-E). BFU-E cells represent the most proliferative differentiation stage, followed by CFU-E cells. During this period, the



**Fig. 1** Morphological and structural changes during erythropoiesis. **a** Erythrocytes are originated from undifferentiated progenitors, burst forming units of erythrocytes (BFU-E) and colony forming units of erythrocytes (CFU-E), located in the bone marrow of adult mammals. Differentiation of CFU-E generates a succession of precursors, proerythroblasts (PRO), basophilic erythroblasts (BE), polychromatophilic erythroblasts (PE), and orthochromatic erythroblasts (OE) in a event organized on a central macrophage. OE is partitioned in two structures, reticulocytes (R) and pyrenocyte (P) throughout the enucleation process, the pyrenocyte is phagocytosed by the central macrophage, and the reticulocyte is turned over to the blood where the maturation of the erythrocyte is completed. The arrows indicate the sequence of differentiation. During erythropoiesis, important changes in the cytoskeleton of tubulin is observed. In PRO, a typical web of microtubules is seen. During enucleation (EN), the nucleus migrates to one side of the cytoplasm to establish polarity, which is mediated by regulated microtubules. After nucleus removal, a higher content of tubulin is retained in reticulocytes, and it is observed as monomers of tubulin dispersed in the cytosol. Finally, in mature erythrocytes an important content of tubulin is observed as a macrostructure that sediments to 100,000×g and takes the form of connected rings. A graphic representation (**b**) and fluorescence microscopy (**c**) of the mentioned structures is shown

cells are completely dependent on erythropoietin (EPO) for their survival [18], the self-renewal of CFU-E [19], and their ability to generate colonies in vitro [20]. In fact, mice lacking erythropoietin or its receptor die around embryonic day 13, due to failure of definitive fetal liver erythropoiesis [18, 21]. Other factors are also necessary to induce the growth of BFU-E and CFU-E, such as some cytokines secreted by macrophages [22, 23].

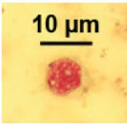
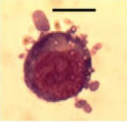
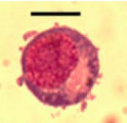
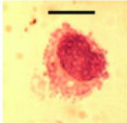
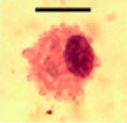
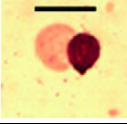
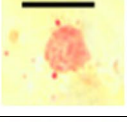
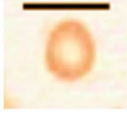
Stress erythropoiesis differs from bone-marrow steady-state erythropoiesis. Steady-state erythroid progenitors

have been shown to develop from a defined hierarchy of myeloid differentiation [24, 25] but stress erythroid progenitors in the spleen are derived directly from short-term reconstituting hematopoietic stem cells (ST-HSCs, CD34 + Kit + Sca1 + Lin<sup>-</sup>), which migrate to the spleen. Once in the spleen, these cells become committed to the stress erythroid fate and undergo an initial expansion stage where cells maintain their stem cell characteristics but are unable to differentiate. The increase in serum EPO concentration causes the progenitor cells to transition to committed erythroid progenitors, which will form BFU-E colonies when plated in methylcellulose media [26]. Another

feature of stress erythropoiesis is that it is not continuous like steady-state erythropoiesis. It occurs in a coupled wave of expansion, transition, and differentiation. Afterwards, new cells need to migrate into the spleen to start the cycle again [26–28]. Finally, unlike steady-state BFU-E formation, which requires two EPO signals and a second signal (a burst promoting activity), stress BFU-E formation requires only EPO.

In steady-state erythropoiesis, differentiation of erythroid progenitors involves the synthesis and expression of major cytoskeletal proteins found in the human erythrocyte membrane. These changes are progressive and can be

**Table 1** Skeleton characteristics in differentiating human erythroid cells

Cell	OM	Skeleton characteristic	References
BFU/CFU		Asynchronous expression of major cytoskeletal proteins starting by spectrin. Presence of F-actin. Tubulin observed as a thin peripheral ring.	[29], [30], [31], [34].
Pro-erythroblast		Low levels of components of the membrane skeleton, $\alpha$ - and $\beta$ -spectrin, adducin, and tropomodulin. High levels of actin and presence of a well-defined typical microtubule network	[30], [34], [41], [42].
Basophilic erythroblasts		Increases at later stages of differentiation of $\alpha$ - and $\beta$ -spectrin, adducin, and tropomodulin. Presence of proteins that link the lipid bilayer to the skeletal protein network (band 3, RhAG, ankyrin, and 4.1R). Decreased levels of actin.	[34], [41], [42], [54].
Polychromatic erythroblasts		Loss of structure of microtubules, presence of disorganized pattern of tubulin around nucleus.	
Orthochromatic erythroblasts			
Enucleation		Event dependent on actin polymerization, microtubule polymerization and vesicle trafficking and subsequent vacuole coalescence.	[36], [45], [46], [48], [54].
Reticulocyte		Structured scaffold of tetrameric spectrin. Elevated incorporation of the actin- and 4.1R binding spectrin fragment into the membrane. Significantly amount of tubulin.	[34], [48], [61].
Erythrocyte		Scaffold of tetrameric spectrin in association to actin, ankyrin, 4.1 R, tubulin. Interaction of skeleton to the membrane by band 3, glycoforin C and others. Presence of intermediate filaments in zones of high physical pressures. Different tubulin isotypes distributed in membrane, cytosol and sedimentable fraction.	[81], [91], [92], [96], [103], [107], [108], [109], [123].

Different intermediate stages of erythropoiesis and their main skeleton characteristics are shown. Cells were observed by optic microscopy (OM)

observed from the beginning of the process (Table 1). As soon as 3 days in the BFU-E culture, spectrin can already be detected. This is earlier than the synthesis of hemoglobin and coincides with the time when BFU-E becomes EPO-dependent [29]. Actin is also present in BFU-E and CFU-E. F-actin bundles are identifiable in the cytoplasm of CFU-E [29], and nucleation of this F-actin in HSCs involves the activity of different members of the Rho family [30, 31]. The rearrangement of cortical actin is essential for normal erythropoiesis. Defects in the assembly of F-actin are related to a deficiency in adhesion and migration, as well as to the defective engraftment of progenitors [30, 32]. Consistently, there have been reports on a comprehensive transcriptional deregulation of pathways and single genes associated with the organization of the cell cytoskeleton, cell migration, and cell adhesion in the HSPC subsets of multiple myeloma patients [33].

On the other hand, there are no reports on the structure and dynamism of tubulin in erythroid progenitors. Recent work by our group showed that tubulin was present in hematopoietic progenitor stem cells (HPSC) (CD34+, lineage-) from umbilical cord blood. Fluorescence corresponding to tubulin was observed in these cells in the form of a thin peripheral ring, because the cells were small, with very low activity and little cytoplasm. Hemoglobin was absent at this stage [34]. No distinction was made in this work between BFU-E or CFU-E (Table 1).

In the second phase of erythropoiesis, CFU-E cells undergo mitosis to generate proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, and finally orthochromatic erythroblasts, which suffer enucleation to produce reticulocytes and eventually complete their maturation into erythrocytes one to 2 days later (Fig. 1a). In the late polychromatic erythroblast stage, the cells survive independently of EPO [35, 36]. The process of erythroblast maturation is characterized by a progressive decrease in cell size, a loss of cytoplasmic basophilia partly due to the hemoglobin accumulation, the elimination of intracellular organelles (e.g. the Golgi apparatus and the endoplasmic reticulum), cytoskeleton remodeling, nuclear condensation, loss of cytoplasmic-nuclear junctions, and ultimately nuclear extrusion, which is the last step in mammals only. In this phase of erythropoiesis, erythroblast interactions play crucial roles [37]. It has been demonstrated that erythroblast proliferation increases threefold when these cells interact with macrophages [12]. Erythroblast-erythroblast and erythroblast-extracellular matrix interactions are also significant. Homotypic signaling between erythroblasts is necessary to regulate gene expression [38]. Moreover, fibronectin and laminin, two matrix proteins, are possible regulators in the terminal differentiation of erythroblasts and reticulocytes [37].

Interactions between erythroblasts and the matrix or other cells is possible thanks to an adhesive interaction system composed of surface adhesion molecules. While erythroblast macrophage protein (Emp),  $\alpha 4 \beta 1$  integrin, VCAM-1, and erythroid intercellular adhesion molecule-4 (ICAM-4) mediate erythroblast-macrophage binding,  $\alpha 4 \beta 1$  integrin, the blood group antigen Lutheran glycoprotein and EphB1 are involved in erythroblast attachments to fibronectin, laminin, and agrin, respectively [39, 40]. The expression of adhesion molecules is modulated during erythroid cell maturation. They are more strongly expressed in proerythroblasts and either at very low levels or not expressed at all in orthochromatic erythroblasts [41]. For instance, CD44 is 30 times lower in proerythroblasts than in orthochromatic erythroblasts [42], which are why this protein is used as a marker in flow cytometry and immunoblotting analyses [43]. This downregulation of adhesion proteins provides cells with the ability to migrate efficiently from the bone marrow to the periphery.

As opposed to the expression pattern observed for adhesion molecules, the expression of membrane skeletal proteins increases during erythropoiesis (Table 1). The levels of  $\alpha$ - and  $\beta$ -spectrin, whose expression started in the previous phase, increase in the second phase. Other membrane skeleton components, such as adducin and tropomodulin, also start to be synthesized in the second phase, at the proerythroblast stage and progressively increase in later differentiation stages. Proteins that link the lipid bilayer to the skeletal protein network (band 3, RhAG, ankyrin, and 4.1R) occur later, and consequently, the assembly of the spectrin-based network, an important determinant of erythrocyte membrane properties, is a late event in erythropoiesis [41]. The expression of actin follows a different pattern. It is highest in proerythroblasts and decreases as the terminal erythroid differentiation proceeds, possibly because it has a function to perform in erythroblasts, which it probably exercises in its filamentous state in the cytoplasm, whereas only a small proportion is required to form the short protofilaments of the skeletal lattice [41, 42]. Cytoskeleton organization requires additional proteins, such as the regulator cyclin-dependent kinase 6 (Cdk6). Loss of Cdk6 kinase activity in erythroblasts induces a significant decrease in the mRNA levels of Baiap2, Gelsolin, and Pip5k1b, involved in the assembly of actin, and of tubulin  $\alpha$ -8, implicated in the assembly of microtubules. Moreover, Cdk6-deficient cells show lower levels of F-actin and are more sensitive to actin and microtubule inhibitors [44]. A mass spectrometry analysis of erythroid cells confirmed that Cdk6 interacts with a number of proteins involved in cytoskeletal organization, which suggests that Cdk6 fulfills a global role in cytoskeletal integrity [44]. As in the case of progenitors, few studies have reported on the presence and function of cytoskeleton tubulin in erythroblasts. Nigra et al. [34] reported that

tubulin undergoes rearrangements during erythropoiesis in proerythroblasts, which have an active metabolism. Tubulin was observed as a well-defined typical microtubule network, but while differentiation continued, the microtubules were restructured. By day 13 of HPSC incubation, when late orthochromatic erythroblasts were abundant, tubulin had assumed more disorganized structures located mainly around the nucleus (Table 1).

There are no reports about the remodeling of cytoskeleton during stress erythropoiesis, but given that the progenitors and signals required for this process are different than those used in bone-marrow erythropoiesis and that erythropoiesis is dynamically orchestrated by multiple genes and regulatory proteins, defects in the integrity of the cytoskeleton, such as those observed in different types of anemia, could result from the differences between both processes.

Erythropoiesis in mammals concludes with the dynamic process of enucleation, whereby the orthochromatic erythroblast generates a reticulocyte that matures into a red blood cell and a pyrenocyte, which is a membrane-encased nucleus surrounded by a thin rim of cytoplasm.

## Involvement of cytoskeleton in enucleation

Before enucleation, erythroblasts undergo several changes, such as a decrease in cell size, nuclear condensation, and loss of cytoplasmic-nuclear junctions, which are necessary to generate an extremely asymmetric division in preparation for enucleation. Afterwards, reticulocytes mature into erythrocytes in the bloodstream, and pyrenocytes are phagocytosed by central macrophages (Fig. 1a).

Enucleation is not completely understood because it is very dynamic and complex [45]. Two models were put forward to explain the extrusion of the nucleus: apoptosis and asymmetric cytokinesis. The former was discarded because caspase inhibitors are not able to block enucleation [46]. In the latter, supported by many studies, nuclear extrusion occurs when the nucleus is segregated from the cytoplasm by an active process that involves cytokinetic machinery [36]. Pyrenocytes are surrounded by an intact plasma membrane [46, 47], which confirms that the nucleus is separated in a well-orchestrated process. Moreover, a cytoplasmic constriction can be seen during enucleation between the incipient reticulocyte and the pyrenocyte in the cleavage furrow, in the equatorial region of a mitotic cell. This event depends on actin [48] and microtubule polymerization [49], as well as on vesicle trafficking and subsequent vacuole coalescence, which provide additional membrane for the separation of nucleus and reticulocyte [50] (Table 1).

One of the first events during enucleation is the establishment of cell polarity when the nucleus migrates to one side of the cytoplasm. According to several reports, microtubules

are required in this process. Chasis et al. [49] demonstrated that colchicine, a polymerization microtubule inhibitor, blocked the enucleation process both *in vitro* and *in vivo*. Images obtained through flow cytometry and fluorescence microscopy showed that tubulin creates a cage-like structure around the nucleus before enucleation (Fig. 1b, c). Treatment of the orthochromatic erythroblasts with colchicine disrupts this structure, leading to a diffuse microtubules distribution pattern [45, 51]. Later, Nigra et al. [34] demonstrated that during enucleation tubulin clearly tends to move towards the side of the cell opposite the nucleus. Even though enucleation depends on microtubules, microtubule-organizing centers (MTOC) are not involved in the process. The treatment of erythroblasts with different MTOC inhibitor regulators reported by Kobayashi et al. [52] did not block enucleation. Nuclear positioning and microtubule organization are both regulated by dynein, whose elimination by Trim58, an E3 ubiquitin ligase superfamily member, is necessary to detach microtubules from the cell cortex and the nuclear membrane. These events promote microtubule reorganization and nuclear polarization [46, 53]. During erythropoiesis, microtubules also regulate the activity of phosphatidylinositol-3-kinase (PI3K). When late-stage erythroblasts were treated with nocodazole, a microtubule-disrupting drug, PI3K products could not be found in the plasma membrane but rather diffusely located. Ji [54] proposed that microtubules indirectly control PI3K activity to regulate the contractile actin ring formation and enucleation. This is backed up by the fact that both PI3K inhibition and depolymerization microtubule caused impaired cell polarization, leading to a severe delay in enucleation [18, 51].

There is some evidence that intermediate filaments are not involved in enucleation since the expression of vimentin is downregulated as erythropoiesis progresses [48]. However, vimentin loss may play an important role in preparing the cells for enucleation [55], since it may prompt the liberation of the nucleus and the establishment of nuclear polarity [54, 56].

The third component, actin, has a role to play as well [36, 46, 48]. When the nucleus is displaced to one side of the cell, F-actin is restricted to the other side, near a constriction zone: these narrow constrictions on the cell surface create enough pressure to push out the nucleus, thus forming a bud [45, 48]. Accumulation of actin in this region is mediated by activation of the Rac GTPases Rac1 and Rac2 [41]. A Rho-effector, mDia2, was also found to be essential for erythroblast cytokinesis since its deficiency in mice caused the failure of primitive erythropoiesis and embryonic death [57]. Furthermore, phosphorylated myosin regulatory light chain (pMRLC) colocalized with actin in the same area [45], and Ubukawa et al. [58] showed that the myosin inhibition by blebbistatin, a non-muscle myosin II ATPase inhibitor, blocks enucleation, which suggests that enucleation

could not take place without non-muscle myosin II. Taken together, these results indicate that a functional actomyosin ring is formed during enucleation. At the final stage of terminal erythropoiesis, the congregation of mitochondria, endocytic vesicle trafficking, and vacuole formation were reported to be in the area between the incipient reticulocyte and pyrenocyte. The authors suggested that the function of mitochondria is to supply enough energy for enucleation to proceed, while vesicles provide the limiting membrane for the incipient nucleus [47, 54]. Vesicle trafficking seems to depend on clathrin, since the inhibition of clathrin-dependent vesicle trafficking blocked enucleation of primary fetal liver erythroblasts without affecting normal differentiation or proliferation, although it is not clear if there is cross talk between the actin cytoskeleton regulatory machinery and this trafficking [50]. Interestingly, the expression of neuronal protein  $\alpha$ -synuclein increased during terminal differentiation of erythroblasts, and Araki et al. [59] suggested that this protein is involved in the stabilization of the erythroid membranes. Other authors also found that lipid rafts are distributed between the nascent reticulocyte and pyrenocyte, suggesting a functional role for lipid rafts in enucleation [60].

An important event during enucleation is membrane remodeling, in which components are sorted to the plasma membranes between the reticulocyte and the pyrenocyte, and the linkages between continuously expressed proteins are modified. It has been reported that several skeletal elements, such as actin, spectrin, tubulin, ankyrin, and protein 4.1 are partitioned to young reticulocytes [48, 61], but the molecular mechanisms underlying protein sorting have not been elucidated. Lee et al. [61] proposed that skeletal linkage might control the sorting pattern of transmembrane proteins. In fact, the authors demonstrated that glycophorin A (GPA) molecules are predominantly connected to the membrane skeleton late in terminal differentiation and are partitioned almost exclusively to the reticulocyte during enucleation. These data are in agreement with earlier studies by Skutelsky et al. [62], who reported that non-sialylated glycoproteins are enriched in pyrenocyte membranes, while sialoglycoproteins, such as GPA, are enriched in reticulocyte membranes.

## Mechanical changes during reticulocyte maturation

Reticulocyte maturation is the final step of terminal erythroid differentiation. It starts in the bone marrow, and between 24 and 48 h later the reticulocytes are expelled into circulation to complete the process. During maturation, there are changes in their protein content and membrane organization, which in turn induce changes in cell shape and the mechanical functioning of the membrane. During this

late step, a shape transition from multilobular to biconcave is seen and the deformability and stability of reticulocytes increase as they mature [56, 63, 64]. The cell evolves from an adherent, immature reticulocyte with cytoplasmic organelles into a nonadherent erythrocyte devoid of organelles, with mechanical characteristics that allow it to move through the capillaries.

An important change during reticulocyte maturation is the release of small vesicles called exosomes. The reticulocyte membrane has a larger surface area than that of the erythrocytes, causing the cytoskeleton to be further extended and inducing a compressive force higher than that measured in the erythrocytes. Using a coarse-grained molecular-dynamics reticulocyte membrane model, it was demonstrated that this compressive force is uniformly applied on the lipid bilayer when the cytoskeleton connectivity is high, but when connectivity is reduced, as it is in reticulocytes, the force is not uniformly applied. Therefore, the lipid particles are forced to migrate to the locations where the actin-spectrin connections are removed, leading to detachment of the lipid bilayer from the cytoskeleton and membrane budding. Then, weak interactions between the actin junctions and spectrin filaments could be responsible for releasing vesicles during the passage of reticulocytes in the microcirculation [65]. Exosome release contributes to cell surface decrease and shape transition from multilobular to biconcave [65–67] and could be an important pathway to change the mechanical properties of cells. The decrease in membrane content allows normal erythrocytes to maintain equilibrium between the compressive force exerted by the cytoskeleton on the lipid bilayer and the bending resistance of the lipid bilayer [68].

In addition, it has been reported that a marked reorganization of the membrane skeleton during reticulocyte maturation regulates deformability and stability properties [64, 65, 69–71]. The surface area of reticulocytes is larger than that of erythrocytes, whereas there is a small difference in the cytoskeletal proteins between these two groups. As a result, the cytoskeleton of the reticulocyte membrane could be under tension, leading to increased shear stiffness of the cell membrane. Using coarse-grained molecular-dynamics reticulocyte membrane model, where spectrin length was proportionally varied, demonstrated that elongated spectrin filaments can elevate the shear modulus of the cell membrane, thus providing a possible explanation for the increased stiffness of reticulocytes [65]. Moreover, imaging of the cytoskeleton from AFM revealed that the spectrin filaments in immature reticulocytes were elongated, compared to the mature RBCs [72].

It was also proposed that changes in the mechanical properties between reticulocytes and erythrocytes would be a consequence of changes during maturation reticulocytes in the synthesis of other cytoskeletal proteins. Among them, the synthesis of protein 4.1 is a late event in erythropoiesis

[41]. This protein is an important component due to its horizontal and vertical interactions within the skeletal network. Thus, protein 4.1 facilitates spectrin-actin interactions and the binding between the skeleton and the lipid bilayer through the cytoplasmic domain of the integral proteins, such as glycophorin C and band 3 [70]. Then, the synthesis of protein 4.1 and the establishment of their interactions would be involved in the increase in stability of mature erythrocytes. Throughout differentiation, the expression of protein 4.1 switches due to changes in splicing. The expression of protein 4.1 with spectrin-actin binding domain is a very late event during the erythropoiesis. Since this domain plays a critical role in regulating membrane material properties, this switch may be crucial to the reorganization of the skeletal network [70].

Another protein expressed in the late erythropoiesis is glycophorin C, responsible for tethering the cytoskeletal network to the lipid bilayer via additional binding proteins, such as protein 4.1 and adducin [73]. Then, the deficit of either protein 4.1 or glycophorin C could result in a weak cohesion between the lipid bilayer and the cytoskeleton and consequently in the instability of the reticulocyte membrane. In fact, the junctional complex organized around protein 4.1R is more labile in reticulocytes than in mature erythrocytes [49, 56]. In concordance, the amount of energy needed to dissociate the lipid bilayer from the cytoskeleton of immature reticulocytes was twice as small as that of the mature erythrocytes, suggesting that a weakened association occurs between the cytoskeleton and the lipid bilayer or within the cytoskeleton of the young reticulocytes, where vertical and horizontal interactions are not completely established [71]. Moreover, instability of the cell membrane is observed in some blood disorders, such as hereditary spherocytosis, hereditary stomatocytosis, and hereditary elliptocytosis, where protein defects in the vertical and horizontal interactions were demonstrated [67, 74]. All these pathologies have in common the fact that they present alterations in some cytoskeleton component, and consequently alterations in the morphology and mechanical properties of erythrocytes were observed, for example, in hereditary spherocytosis, quantitative or qualitative defects were observed in ankyrin, protein 4.2, band 3 protein,  $\alpha$ -spectrin, and  $\beta$ -spectrin, which are encoded by the ANK1, EPB42, SLC4A1, SPTA1, and SPTB genes, respectively [75]. On the other hand, in hereditary elliptocytosis, heterozygous mutations of genes that encode the  $\alpha$ -spectrin (encoded by SPTA1),  $\beta$ -spectrin (encoded by SPTB), or protein 4.1 were reported [76]. Finally, hereditary stomatocytosis is a disorder deriving from Band 3 mutations, which affect protein folding and interactions [77]. Cell deformability is another mechanical property that is increased during reticulocyte maturation. The elastic resistance of the membrane skeleton must be high enough to maintain cell cohesion under the shear stresses encountered

in the vasculature, but low enough to enable the mature erythrocyte to deform easily within its constraints of fixed surface area and volume. Then, membrane deformability depends on the flexibility of the skeletal network [69, 71]. Therefore, to allow proper deformability, the skeletal network must be able to undergo rearrangement, and the spectrin molecules must be able to fold and unfold. Changes in intermolecular or intramolecular associations of the skeletal proteins or an increased association of integral membrane proteins with the skeletal network would have a profound effect on membrane deformability [65]. As in the case of stability, changes induced in the cytoskeleton during the reticulocyte maturation could be responsible for changes in this mechanical property. In fact, the results of Li et al. [65] strongly suggest that different skeletal protein interactions regulate the deformability and stability of erythrocytes. Points of association between spectrin with other proteins such as actin, protein 4.1, or tubulin would be essential to regulate cell deformability.

Understanding protein dynamics during the final phases of erythropoiesis could shed light on pathological conditions whereby RBC maturation is impaired, such as reticulocytosis and related anemia [72].

## Cytoskeletal components in erythrocytes

As mentioned above, erythrocytes are the main cellular component of whole blood, about a thousand times more concentrated than white blood cells and platelets, with their volume fraction (hematocrit) being as high as 45%. In mammals, erythrocytes are devoid of nuclei and other cellular organelles, while non-mammalian erythrocytes (birds, fish, and amphibians) are nucleated. The main function of erythrocytes is the transport of oxygen to enable cell respiration. Thus, two of the main requirements for them to be viable and circulate through the vessels are sufficient deformability and mechanical stability. In fact, mature erythrocytes are able to undergo large passive deformations during their passage through vessels with cross-sections one-third their own diameter. The most dramatic manifestation of this property is the passage of erythrocytes from the splenic cords to the splenic sinus, and during flow-induced deformation *in vitro* [78]. Indeed, alterations in the mechanical behavior of erythrocytes have been reported as the underlying cause of many forms of hemolytic anemia [43, 51]. Moreover, microcirculatory disorders such as diabetes mellitus, hypertension, and hemoglobinopathies feature altered erythrocyte deformability [2, 34, 79, 80].

The main factors that determine erythrocyte deformability are the fluid cell membrane and the structure of the membrane cytoskeleton. However, certain intracellular components take part in or can even be fundamental

to the maintenance of the cell biconcave shape [81], since the fresh erythrocyte ghost, consisting only of a membrane and a membranous cytoskeleton, takes on a spherical shape instead of the peculiar biconcave one. Deformability is also modified by the resistance of the cytosolic pool, which is determined by intracellular viscosity, erythrocyte hydration state, and surface-volume interaction [82], as well as by metabolic processes that control ATP levels and the redox state of cells [83].

The plasma membrane of mammalian erythrocytes is the only structural component, and it is responsible for transport and the mechanical characteristics of these cells [84]. It is composed of a fluid bilayer of lipids, mainly cholesterol, asymmetrically distributed phospholipids, and more than 50 transmembrane proteins. Although proteins are mainly in charge of transportation across the membrane, several of them also have a structural function, usually performed by an intracytoplasmic domain interacting with cytoskeletal proteins [85, 86]. Underneath the lipidic bilayer, there is a two-dimensional scaffold known as the spectrin-based membrane skeleton or network. It is connected to the bilayer by integral and peripheral membrane proteins. The connections between membrane proteins and this network are known as vertical interactions, while those between the components of the network itself are referred to as horizontal interactions [87]. Disruptions in the vertical or horizontal interactions induce changes in network density and, consequently, in cell deformability [88]. Studying erythrocytes through microscopy is very difficult due to the high hemoglobin content [89], so there are few reports on the cytoskeleton of the enucleated erythrocyte [81]. Nevertheless, new technologies have made substantial advances possible in this field. The spectrin network has been extensively studied, because spectrin is known to contribute to both the establishing and maintaining a diverse specialized plasma membrane domain, not only in erythrocytes but also in all the animal tissues examined, with their presence being likely in all metazoan cells [90]. The network is organized by the interaction between two spectrin heterodimers, consisting of  $\alpha$ - and  $\beta$ -subunits of 280 and 247 kDa in size, respectively [91, 92]. The heterodimers interact with each other in a head-to-head manner to form antiparallel heterotetrameric filamentous units [93]. Cryo-electron tomography has shown that although the tetramer is the most prominent conformation of spectrin, the complexes can also include hexamer and octamer spectrin oligomers [94, 95]. Spectrin self-association is relatively weak in the erythrocyte [96]. This mechanism seems to be an evolutionary adaptation that allows tetramers to dissociate and reform when the membrane is distorted by shear forces [97, 98].

As mentioned above, spectrin biosynthesis occurs in the early stages of differentiation [29]. Erythroid  $\beta$ -spectrin expression *in vivo* may be induced after progenitor stem

cells have progressed to the myeloid stage and before differentiation to the CFU-E stage. Thus expression of this protein may provide an early membrane marker of erythroid differentiation several divisions prior to the expression of the globin genes [99]. The synthesis of spectrin continues during erythropoiesis. In fact, most of the spectrin that accumulates in enucleating reticulocytes is synthesized during the last few days of this process [100]. Pulse-labeling of nucleated red cell precursors shows that the newly synthesized  $\alpha$ - and  $\beta$ -spectrins are present in the cytosol, with a several-fold excess of  $\alpha$ -spectrin over  $\beta$ -spectrin. However, in the membrane-skeletal fraction, newly synthesized  $\alpha$ - and  $\beta$ -spectrins are assembled in stoichiometric amounts, suggesting that the association of  $\alpha$ -spectrin with the membrane skeleton may be rate-limited by the amount of  $\beta$ -spectrin synthesized. Hanspal and Palek [101] demonstrated that, in rat nucleated red cell precursors, the newly synthesized  $\alpha$ - and  $\beta$ -spectrin of the cytosol turn over coordinately and extremely rapidly. The newly synthesized spectrin may be attached to the plasma membrane before proteins 2.1 and 4.1 are completely synthesized and incorporated into the membrane [100].

Three to six spectrin tetramers bind at either end of the tetramer to one actin protofilament, composed of 12–14 actin monomers, to form the horizontal axis of a structure known as the junctional complex, leading to a pseudo-hexagonal arrangement [102]. The interaction between spectrin and actin is possible due to the  $\beta$ -spectrin present in their N-terminal, which has an actin-binding domain (ABD) or a 2CH domain [90, 103]. Such organization could be dependent on Rac GTPases and Hem-1, a member of the Wiskott-Aldrich syndrome verprolin-homologous (WAVE) protein complex that regulates F-actin polymerization, since mice that lack these proteins develop hemolytic anemias with misshaped erythrocytes and clumped or irregular skeletons [104, 105]. The spectrin-actin interaction also requires the presence of other proteins: the erythroid isoform of protein 4.1, p55/MPP1, protein 4.2, and several actin-binding proteins such as dematin, adducin, tropomyosin, and tropomodulin. All of them are important since they increase spectrin-actin affinity or regulate the interaction between the proteins that form the complex [96, 103, 106–109]. On the cytosolic side of the erythrocyte plasma membrane, the spectrin network is tethered to ankyrin, which couples spectrin to band 3, and to protein 4.1R, which couples the junctional complex to glycophorin C [95, 110]. These vertical interactions may help to regulate cohesion between the lipid bilayer and the membrane skeleton and thus enable the erythrocytes to maintain their favorable membrane surface area. Besides ABD domains, the spectrin structure is reported to have SH3, EF-hand, and PH domains, as well as a CCC region and ankyrin-binding sites. These multiple domains allow spectrin to interact with a broad range of proteins and



phospholipids and, consequently, establish vertical interactions [90, 111–115]. A multiprotein complex, where multiple interactions are established between proteins, mediates the binding of spectrin with the plasma membrane through ankyrin. Band 3, glycophorin A or B, protein 4.2, and the Rh complex are members of this complex. Other proteins such as CD47, the Landsteiner–Wiener blood group antigen, and the proteins in the glycolytic metabolon are also present, but in lower amounts and not in all the complexes. Like the ankyrin complex, the actin junctional complex is centered on band 3. Protein 4.1R and ankyrin compete for binding to this band, which suggests they bind to separate band 3 populations. Protein 4.2, adducin, demantin, glycophorin A, glycophorin C/D, glucose transporter 1, and stomatin are part of this second complex. Due to the physical proximity between both complexes, it is likely that they collide during cell deformation and, therefore, exchange some common proteins such as band 3, protein 4.1R, protein 4.2, and adducin [98].

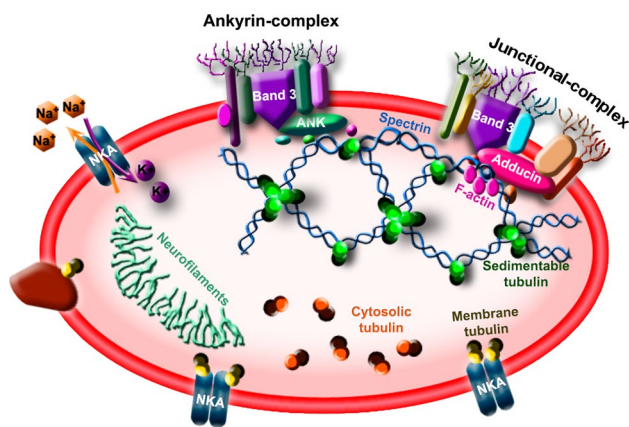
Terasawa et al. [81] used ion-etching/scanning electron microscopy and saponin-ethanol treatment combined with immunocytochemistry to identify a cytoplasmic endoskeleton apart from the classical membranous cytoskeleton in the human erythrocyte. Analysis of the three-dimensional structure showed that the actin-rich endoskeleton is divided into two layers, a superficial one where filaments are perpendicularly connected to the membranous cytoskeleton, and a deeper one where filaments form an irregularly directed, complex meshwork. In the transitional hillside region between the convex periphery and the concave center, the endoskeletal filaments containing a neurofilament protein run parallel to the hillside slope toward the center. This endoskeleton could be associated with the membranous cytoskeleton, influencing the biconcave shape by making it deformable, pliable, and restorable against external circumstances. The same report identified the presence in erythrocytes of a kind of intermediate neurofilament protein, the second component of the cytoskeleton. This protein is scarce in the concave center but very prominent in the hill of the cell, where physical pressure is applied during erythrocyte deformation, suggesting that neurofilaments play an important role in stabilizing the biconcave shape. The presence of these structures is not surprising, given that before enucleation, the immature mammalian erythroblasts contain cytoskeletal structures that are similar to those of non-mammalian vertebrate erythrocytes.

The presence of a microtubule network, the last component of the cytoskeleton, is unclear in mammalian erythrocytes. For many years, it was thought that tubulin was lost with the nucleus during enucleation. Moreover, van Deurs and Behnke [116] reported an absence of microtubules in human erythrocytes, and Liu et al. [56] showed that tubulin is lost during reticulocyte maturation. Nevertheless, there is

currently evidence that tubulin is part of both the endoskeleton and the erythroid membrane. Simpson and Kling [47] described the microtubule presence in circulating erythrocytes, though at low levels. A more recent study on the proteomic profile of membrane protein distribution in sorted populations of reticulocytes and extruded nuclei reported the presence of tubulin as well. In this study, proteins were fractionated by 1D SDS-PAGE and subjected to Nano LC mass spectrometry. After enucleation, significant quantities of tubulin  $\alpha$ -1,  $\alpha$ -8, and  $\beta$ -6 chains were observed in reticulocytes. In all cases, the total number of peptides was higher in the reticulocyte population than in the extruded nuclei [117]. This coincides with the study by Nigra et al. [34], who reported that tubulin is retained in reticulocytes following enucleation. These reports confirm that many erythroid membrane and cytoskeletal proteins partition predominantly or exclusively to the reticulocyte during erythroblast enucleation alongside the expected cytosolic, mitochondrial, endosomal, and lysosomal proteins. In contrast, nuclear proteins, ER proteins, and a contingent of cytosolic and plasma membrane proteins are distributed with the extruded nucleus [118].

Although a large amount of tubulin is lost during the erythrocyte maturation process [56, 119], some tubulin can be found in mature erythrocytes. A proteomic analysis demonstrated that these cells possess proteins related to cytoskeletal proteins, including MAPs, two alpha chains ( $\alpha$ -6 and  $\alpha$ -3), and one beta chain ( $\beta$ -1) of tubulin, 14-3-3 protein tau, similar to fibronectin type 3 protein, a SPRY domain-containing protein, and different actin isoforms [120–122]. Previous studies by our group confirmed the tubulin presence in these cells. Amaiden et al. [123] observed through immunoblot and immunofluorescence that erythrocytes possess  $\alpha$  and  $\beta$  tubulin. Tubulin is not a major protein in erythrocytes in terms of volume, but it plays an important structural role and is involved in the regulation of their mechanical (deformability, osmotic fragility) and physiological (ion transport, modified enzyme activity of P-ATPases) properties [34, 80, 89, 123–126].

Three isoforms of tubulin, which are a product of post-translational modifications of the protein, have been reported in erythrocytes (Fig. 2): detyrosinated, tyrosinated, and acetylated [123]. Detyrosinated and tyrosinated tubulin are part of the tyrosination/detyrosination cycle, and the balance between these two isoforms is a consequence of the two enzymes activity: tubulin tyrosine carboxypeptidase (TTCP), which removes the carboxy-terminal tyrosine of tubulin to produce detyrosinated tubulin [127]; and tubulin tyrosine ligase (TTL), which can re-add the tyrosine residue [128]. Both enzymes are present and active in erythrocytes and equilibrium between both is essential to normal cell development. In fact, alterations in the content and activity of TTL produce a detyrosinated tubulin increase and a



**Fig. 2** Different components of cytoskeleton are present in erythrocytes. Erythrocytes possess a spectrin network underneath the membrane, which is important in establishing vertical interactions with the plasma membrane and horizontal interactions with other members of the network. These interactions allow the cells to perform important functions. The spectrin network is anchored to the plasma membrane at two points, in the ankyrin-complex and in the junctional complex, both of which center in the Band 3 protein. The conformation of the latter complex requires the presence of actin protofilaments, composed of 12–14 F-actin monomers, which is the basis for the pseudo-hexagonal arrangement observed in the spectrin network. Spectrin is also associated with tubulin, the second cytoskeletal component. Interactions of both proteins were observed in a structure of tubulin sedimentable at  $100,000\times g$  and sensible to nocodazole. Erythrocyte tubulin was also observed in the cytosol and associated with membrane proteins such as NKA. Finally, neurofilament proteins, a class of intermediate filaments were observed in the region between the convex periphery and the concave center of erythrocytes

$\text{Na}^+$ ,  $\text{K}^+$ -ATPase (NKA) activity decrease. These modifications are related to changes in erythrocyte deformability and hypertension development [126]. On the other hand, the acetylated tubulin content results from the interplay between acetylase and deacetylase enzymes. Reports by our group suggested that erythrocytes have high deacetylase activity and that inhibition of these deacetylases increases acetylated tubulin levels. This triggers various physiological events that affect both enzyme activities and the membrane rheological properties [89]. Alterations in the proportion of these tubulin isotypes are seen in the erythrocytes of hypertensive and diabetic subjects [34, 80, 89, 123–126].

Although tubulin is mainly a cytosolic protein, the use of differential centrifugation and immunofluorescence made it possible to demonstrate that erythrocyte tubulin is distributed into three different intracellular pools (Fig. 2): membrane-associated tubulin (Mem-Tub), cytosolic (soluble) tubulin, and a sedimentable fraction at  $100,000\times g$  (Sed-Tub) [123]. Association of tubulin with the plasma membrane is possible due to its interaction with some integral membrane proteins such as NKA and PMCA [123, 125, 129], but its structural and functional properties in the Sed-tub fraction remain largely unknown. Recently, we used transmission

electronic microscopy to show that even though typical microtubules are not found in the Sed-tub fraction, tubulin is part of a macrostructure that takes the form of connected rings. The Sed-tub fraction behaves similarly to microtubules when responding to taxol and nocodazole, drugs that assemble and disassemble microtubules, respectively. The use of these drugs demonstrated that tubulin plays an important role in the structural organization of the fraction since its absence results in organization loss [34]. Nevertheless, the low proportion of tubulin in the fraction suggests that it interacts with other proteins. In fact, 2-D electrophoresis and MALDI-TOF-MS analyses showed that actin, spectrin, and band 3 are major protein components of this fraction in erythrocytes. In addition, tubulin was observed to physically interact with at least one of these proteins, spectrin [34]. The model proposed by Nigra et al. [34], then, suggests that the fraction displays a non-uniform connected-ring web, with tubulin located in the connection areas, most likely through the interaction with other proteins (e.g. spectrin).

Tubulin content in each erythrocyte fraction is not static but balanced, mainly between the Sed-Tub and Mem-Tub fractions. This balance can be modified according to the cell need, resulting in both mechanical and biochemical changes. Our previous studies have shown that rheological changes observed in some pathologies such as hypertension and diabetes are associated with altered tubulin content and distribution in both the Mem-tub and Sed-tub fractions of human erythrocytes [80, 89, 125, 126]. The ability of tubulin to affect the rheological and biochemical properties of erythrocytes at low levels suggests that it may function as a molecular signal.

## Concluding remarks

In the past, erythrocytes were seen as simple cells, inert to external stimuli, responsible for transporting oxygen. Currently, it is known that they are the main determinant of the rheological properties of blood, mostly thanks to the interaction between the plasma membrane and the membrane skeleton. Many studies have reported on the structure and composition of a spectrin-actin scaffold and on how these components interact with the plasma membrane. However, many aspects of this structure remain to be explored, since very little is known about the manner in which the other cytoskeleton components (i.e. intermediate filaments and tubulin) fit into this structure and about the vertical and horizontal interactions between these proteins and their contribution to cell functioning. It has been reported that the alterations in isotypes or the distribution of tubulin are related to different pathologies, but more exhaustive studies about the mechanisms involved are needed. Previous works of our laboratory demonstrated that the elimination

of tubulin from the sedimentable fraction leads to disassembly of this structure and consequently to the alteration of mechanical and biochemical properties of the erythrocyte. However, given that the erythrocyte tubulin content is low, tubulin removal is likely to affect other components of the cytoskeleton, probably spectrin. Therefore, it would be interesting to evaluate the possible changes occurring in the cytoskeleton after tubulin removal. Modern techniques for fixation, fracture, and observation of erythrocytes are currently available, such as the ion-etching technique and PHA-E coated coverslip combined with different types of microscopy like SEM, TEM, and AFM [72, 81, 130]. These techniques would make it possible to elucidate a 3D structure and a pattern of interaction between proteins and to know the role of each member of the complexes. Alternatively, analysis of the presence in tubulin of potential interaction domains with proteins such as spectrin, actin, band 3, and others and the presence in spectrin of tubulin-binding domains could provide important information to design new study strategies about the functionality of each cytoskeleton protein. Finally, it has been well established that different types of anemia are a consequence of disorders in the expression of some cytoskeleton proteins, as mentioned above. Then, an analysis of changes in the content and distribution of tubulin in erythrocytes from subjects with these types of anemia could bring important advances in this matter.

Another important point is the origin of membrane skeleton elements. Since erythrocytes lack nuclei, all the proteins they contain originate in their progenitors, so abnormalities in erythrocytes could be a consequence of abnormalities in the progenitors or in the differentiation process. In particular, as mentioned above, enucleation and reticulocyte maturation imposes important changes on the cytoskeleton and, consequently, in the mechanical properties of the cells. A recent study shows that the treatment of erythroblast paclitaxel resulted in suppression of tubulin degradation during erythrocyte maturation and abnormal tubulin retention in mature erythrocytes [119]. Similarly, increases in the membrane tubulin content were detected in erythrocytes from diabetic and hypertensive subjects, which correlated with a decrease in cell deformability [89, 123]. Causes of tubulin retention in mature erythrocytes are unknown; therefore, the study of the interaction of different components of cytoskeleton would be very useful in understanding the abnormalities found in the erythrocyte skeleton of individuals with different diseases such as hypertension, diabetes, and anemia.

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