Review Article

Erythroblast Enucleation

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Even though the production of orthochromatic erythroblasts can be scaled up to fulfill clinical requirements, enucleation remains one of the critical rate-limiting steps in the production of transfusable red blood cells. Mammalian erythrocytes extrude their nucleus prior to entering circulation, likely to impart flexibility and improve the ability to traverse through capillaries that are half the size of erythrocytes. Recently, there have been many advances in our understanding of the mechanisms underlying mammalian erythrocyte enucleation. This review summarizes these advances, discusses the possible future directions in the field, and evaluates the prospects for improved ex vivo production of red blood cells.

1. Introduction

Blood transfusions are a common practice to treat severe anemia and shock. Even though developed countries by and large have sufficient supplies of transfusable blood, an increase in the incidence of complications from allogenic immune reactions provides impetus to search for alternatives that are less immunogenic [1–3]. Moreover, developing and third world countries battle a shortage of blood units for transfusions [4]. One alternative under investigation is the ex vivo derivation of red blood cells from autologous hematopoietic stem/progenitor cells. In addition to enhancing the supply of transfusable blood, this approach may decrease the incidence of allogenic immune reactions in chronic transfusion-dependent patients. Human embryonic stem cells, CD34+ cells from umbilical cord blood, adult hematopoietic stem/progenitor cells, peripheral blood CD34+ cells, or human-induced pluripotent stem cells (iPS cells) can all be used as source for synthesizing RBCs [5]. In all these systems, however, the efficiency of enucleation is low. Enucleation is an important criterion because nucleated erythroblasts are not as efficient in oxygen transport and because they are likely to undergo hemolysis as they traverse through narrow capillaries. Enucleated cells also offer the benefit of lacking DNA and the ability to divide, obviating the risk of introducing a malignancy into the recipients [6, 7].

Enucleation remains one of the critical rate-limiting steps of in vitro RBC synthesis. Lu et al. achieved 60% enucleation of erythroblasts derived from human ES cells (hES cells). The RBCs produced expressed mainly fetal and embryonic hemoglobin and were comparable to normal RBCs in terms of oxygen delivery [8]. Recently, Lapillonne et al. have reported the production of red blood cells from human iPS cells. Although the majority of the culture consisted of orthochromatic erythroblasts, enucleated RBCs accounted for only between 4 and 10% of the culture [9]. Thus, there is a long way to go in terms of achieving 100% enucleation in vitro. This review will summarize the current mechanistic understanding of enucleation, propose a new model, and discuss future research directions.

2. Defining Erythroblast Enucleation

Mammals have evolved to enucleate their erythroblasts while other animals maintain circulation of RBCs with condensed pyknotic nuclei. Of note, however, the circulation of early mammalian embryos includes nucleated primitive erythroid cells. These cells mature in the bloodstream between embryonic days (E) 14.5 and E16.5 of mouse gestation and eventually enucleate likely within the fetal liver [10]. Definitive erythropoiesis, which leads to exclusive

production of enucleated reticulocytes, begins in mid gestation. RBCs derived from definitive erythropoiesis originate in the fetal liver or bone marrow depending upon the age of the fetus [11].

In all cases, erythroblasts are derived from hematopoietic stem cells (HSCs). The first cell committed towards the erythroid lineage is the burst forming unit-erythroid (BFU-E), which further proliferates and matures to the colony-forming unit E (CFU-E). The BFU-E stage is the most proliferative segment of the differentiation program followed by the CFU-E stage. Acquisition of EPO receptors occur in the mid-to-late stage of BFU-E, and by the time these cells reach CFU-E stage maximum numbers of EPO receptors are present on their surface [12]. During this time period, cells are completely dependent on erythropoietin (EPO) for their survival [13]. The CFU-E then undergoes a series of maturational steps named proerythroblasts, basophilic erythroblasts, polychromatic erythroblasts, and eventually orthochromatic erythroblasts, at which point they exit the cell cycle. By the time, the cells reach late polychromatic erythroblast stage the cells are independent of EPO for their survival [14]. Other cytokines and humeral factors that may be required to promote differentiation beyond the polychromatic stage has not been identified although autocrine production of several cytokines have been observed [15, 16]. Whether these cytokines provide the necessary signals for terminal differentiation including enucleation is unclear at this point. Throughout the differentiation program, erythroid progenitors and erythroblasts undergo numerous morphological changes. These include (1) a decrease in cell size, (2) nuclear condensation, and (3) an increase in the cytoplasmic nuclear ratio.

As red cells mature, chromatin becomes condensed, and transcription in general is suppressed. A network of chromatin factors and histone modifying proteins likely contribute to this process. For example, the nonhistone nuclear protein mature erythrocyte nuclear termination stage specific protein (MENT) has been shown to promote chromatin condensation and nuclear collapse at the terminal stage of maturation of chicken erythrocytes [17]. Similarly, the condensin II subunit mCAP-G2 represses transcription by recruiting class I histone deacetylases (HDACs) and promotes terminal differentiation of mammalian cells [18]. During maturation of murine erythroblasts, histone H3(K9) dimethylation was found to increase while histone H4(K12) acetylation was dramatically decreased [19]. Consistent with a requirement for decreased acetylation, treatment of murine erythroblasts with the HDAC inhibitors trichostatin A or valproic acid blocked chromatin condensation and enucleation [19, 20]. Moreover, knockdown of HDAC2 led to a prominent block in condensation and nuclear extrusion. Recent studies have further revealed that downregulation of miR-191, which occurs during normal differentiation, contributes to chromatin condensation by allowing the upregulation of Riok3, an atypical protein kinase, and Mxi1, a c-myc antagonist [21]. Downregulation of myc appears to be required not for cell cycle arrest, but rather to allow for nuclear condensation and histone deacetylation [22]. Together these studies show that chromatin condensation is an integral part of erythroblast maturation.

After exiting the final cell cycle, the nuclei of orthochromatic erythroblasts are polarized to one side of the cell. Eventually these cells enucleate to form reticulocyte and a "pyrenocyte," the extruded nucleus with a thin rim of cytoplasm surrounded by a plasma membrane [10, 23]. Pyrenocytes are engulfed by macrophages in erythroblastic islands within the fetal liver and bone marrow. During terminal differentiation, cells undergo multiple cellular processes including protein sorting, autophagy, membrane maturation, vesicle trafficking, and cytoskeletal remodeling. Important questions to the field include the following (1) To what extent do these processes contribute to enucleation? (2) Which of these steps are required for enucleation? (3) At which point in the differentiation continuum does enucleation start? (4) Can enucleation occur in a cell that is not completely matured, such as a polychromatic erythroblast? (5) Can enucleation proceed before nuclear condensation is completed? At this point, it has not been demonstrated whether enucleation can occur in an immature erythroblast. Based on the model that chromatin condensation is a prerequisite for enucleation [19, 20, 24], however, one would assume that this is unlikely. Examining the nuclei engulfed by macrophages within genetically modified mice that have a block in maturation of late erythroblasts may help answer whether enucleation can take place in erythroblasts other than orthochromatic erythroblasts. Since nuclear condensation and other morphological changes are progressive processes that proceed from proerythroblasts through orthochromatic erythroblasts, and since primitive RBCs and nucleated peripheral blood RBCs of lower animals have condensed nuclei, for the purposes of this review, we will consider enucleation to be a process that begins with polarization of nucleus in an orthochromatic erythroblast.

3. Mechanism of Enucleation

3.1. Apoptosis, Asymmetric Cytokinesis, or Other?. Historically there have been two prominent models of enucleation: apoptosis and asymmetric cytokinesis. The presence of partial karyolysis and leakage of nuclear material into the cytoplasm visualized by electron microscopy favors the apoptosis model [25]. Additional evidence includes the prevailing model that lens epithelial cells and keratinocytes undergo a mechanism similar to programmed cell death to eliminate their nuclei [26, 27]. To directly test this whether apoptosis is required for enucleation, Carlile et al. studied the effect of siRNA-mediated caspase knockdown on enucleation and found that there was a 50% decrease in enucleated cells in the knockdown condition as compared to control. However, the authors noted that maturation was blocked at a stage between proerythroblasts and basophilic erythroblasts, suggesting a role for caspases at an earlier stage of erythroblast development [28]. Furthermore, Krauss et al. observed that critical nuclear structures such as the nuclear matrix protein NuMA (nuclear mitotic apparatus) and the splicing factors Sm and SC35, as well as the

interaction between lamin B with the nuclear envelope and DNA persisted during late erythroblast development prior to enucleation, consistent with an absence of caspase activity [29]. In addition, treatment of enucleating erythroblasts with pan-caspase inhibitors did not block enucleation [23]. Together, these findings strongly suggest that apoptosis is not involved in enucleation per se.

The cytokinetic model posits that nuclear extrusion is a form of cell division, in which the nucleus is separated from the cytoplasm by an active process that involves cytokinetic machinery. By this definition, an enucleating cell should have a well-defined cleavage furrow, a contractile actomyosin ring, and a stage of completion with abscission [30]. Indeed, many studies including ultra-structural observations from the 1960s lend support to this model. For example, Skutelsky and Danon and others noticed that the pyrenocytes had a thin rim of cytoplasm surrounded by an intact plasma membrane [23, 25, 31–34], a finding that confirms that the nucleus is not extruded out or exocytosed, but rather separated in a well-orchestrated process. Moreover, one can see a constricted surface resembling a cleavage furrow on enucleating erythroblasts [32, 33, 35, 36]. Further, there are numerous studies showing that cytochalasin D, an actin-depolymerizing agent which blocks filamentous actin formation, inhibits enucleation of late erythroblasts in-vitro [23, 33, 35, 37]. It has also been shown by immunofluorescence that actin accumulates in the region between the nucleus and the cytoplasm close to an anatomic constriction zone [33, 38, 39]. Finally, elegant studies with murine primary erythroblasts have demonstrated that Rac1 and Rac2 function through mDia2 to contribute to actin accumulation in the constriction zone [36]. While these findings provide strong evidence to support a role for the actin cytoskeleton in erythroblast maturation, it is unclear regarding the specific role actin is playing in enucleation, including whether it interacts with nonmuscle myosin II to form a contractile actin ring similar to cytokinesis (Figure 1).

In addition to the actin cytoskeleton, microtubules play an important role in cell division including cleavage furrow formation [30]. Studies by Koury et al. showed that the inhibition of microtubules with various toxins such as colchicine, vinblastine and taxol did not affect enucleation [33]. Ji et al. showed that enucleation does not depend on RhoA activity, using dominant negative mutants of RhoA and C3 exoenzyme, a specific inhibitor of RhoA [36]. It is well known that RhoA is involved in the formation and ingression of cleavage furrow. RhoA when activated accumulates in the cleavage furrow and further activates downstream effectors including Rho kinase (ROCK), citron kinase, LIM kinase, and formins [40–43]. Hence a lack of role for RhoA and microtubule in enucleation suggests that the anatomical constriction zone that is visible on enucleating cell may not be actually a cleavage furrow.

What about intermediate filaments? Using murine splenic erythroblasts infected with the anemia-inducing strain of Friend virus (FVA), Koury et al. found that erythroblasts approaching enucleation downregulate expression of vimentin [33]. Further, Xue et al., using immunofluorescence techniques, observed that vimentin anchored the nuclear lamina to the center of the cell as well as to the plasma membrane periphery and was expressed during the periods of 12, 24, and 36 hours of FVA in vitro culture but lost by 36–48 hours [39]. This loss of vimentin could release the nucleus and enable actin to push it towards one end of the cell close to plasma membrane. Of note, circulating avian erythrocytes continue to have intact vimentin, which might be one of the reasons why they resist enucleation [44].

To test whether actin functions as contractile actomyosin ring in nuclear extrusion, Keerthivasan et al. treated primary murine and human erythroblasts with blebbistatin at different time points and assayed the effect on cell division and enucleation [34]. Blebbistatin is a specific nonmuscle myosin II ATPase inhibitor that blocks the contractility of the actomyosin ring in cytokinetic cells and results in polyploidy of dividing cells without affecting the formation of actin cytoskeleton [45]. Keerthivasan et al. showed that blebbistatin potently inhibited enucleation when added to cultures at 24 hours, when the majority of cells were undergoing cell division. In this case, blebbistatin resulted in cell cycle arrest and the accumulation of polyploid cells. In contrast, blebbistatin had little effect on enucleation when it was added to cells at 38 hours, a time when the majority of cells are postmitotic [34]. These findings suggest that although actin accumulates in the region between the nucleus and cytoplasm during late stage erythroblasts, its role as a contractile actomyosin ring similar to cytokinesis in enucleation is questionable.

Abscission is the final stage of cytokinesis and involves trafficking of vesicles to the midbody region and fusion of these vesicles to lead to separation of the daughter cells [46, 47]. Throughout cytokinesis, membranes are supplied to the progressing tip of the cleavage furrow and the abscission site through golgi and recycling endosome-derived vesicles [48, 49]. Several lines of evidence support the model that vesicle trafficking directly contributes to enucleation, in large part by providing membranes to facilitate the separation of the pyrenocyte from the reticulocyte. First, electron microscopy has revealed the presence of vesicles and U-shaped tubes in the region between the nucleus and incipient reticulocyte [25, 34, 50]. Second, there is an accumulation of transferrin laden vesicles/vacuoles in the region between the nucleus and the cytoplasm [34, 50]. Third, disrupting vesicle trafficking by a battery of chemical inhibitors inhibited accumulation of those vesicles and blocked erythroblast enucleation [34]. Finally, siRNA-mediated knockdown of clathrin inhibited enucleation of human primary erythroblasts [34]. These findings strongly suggest that vesicle trafficking is a key component of erythroblast enucleation and that at least a part of nuclear extrusion process is similar to abscission (Figure 1).

3.2. Protein Sorting and Enucleation.

An important event during enucleation is the differential sorting of proteins to the pyrenocyte and the reticulocyte. Geiduschek and Singer studied this phenomenon by an immunofluorescence technique and followed the distribution of lectin receptors and spectrin through erythroid differentiation. They found that spectrin completely sorts to the incipient reticulocyte
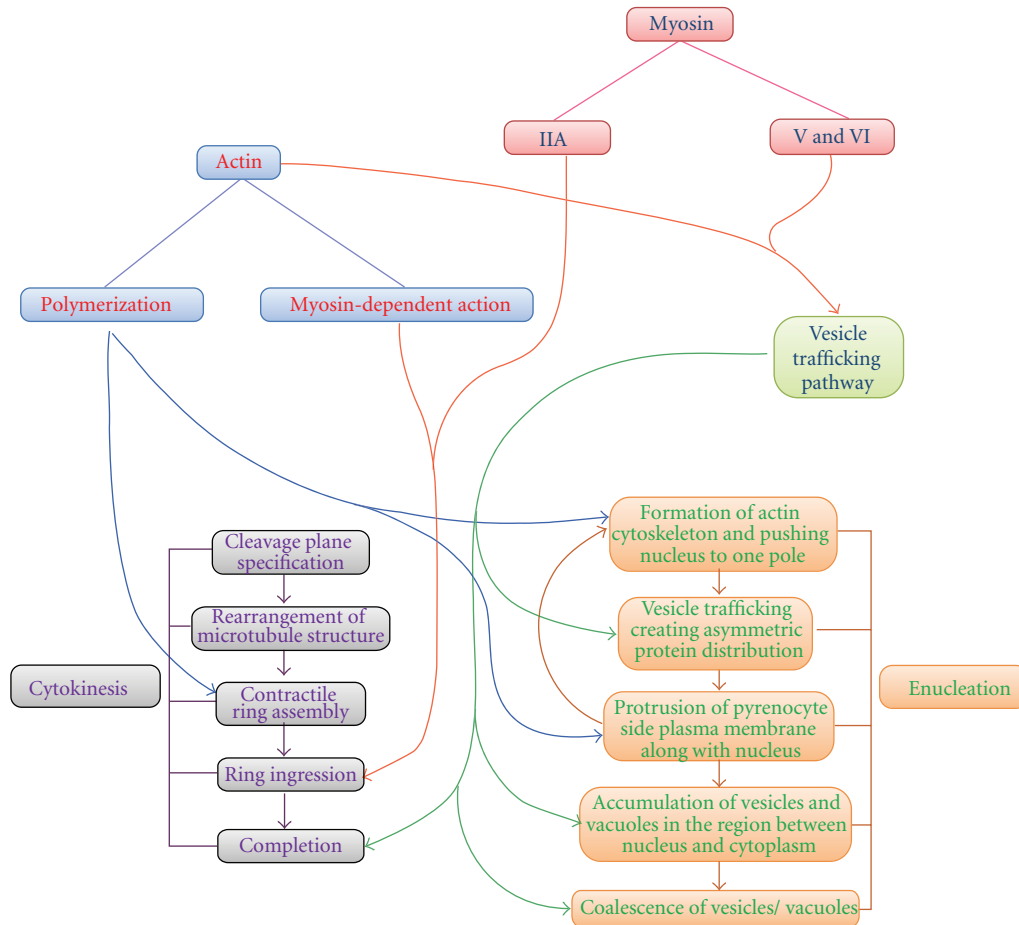
FIGURE 1: Key events in cytokinesis and enucleation. Even though the final stages of cytokinesis and enucleation are both driven by vesicle trafficking, the preceding events are substantially different between the two processes.

while receptors for concanavalin A are restricted to the plasma membrane surrounding the extruding nucleus [51]. Using murine erythroleukemia (MEL) cells, Patel and Lodish reported that enucleated cells detach from a fibronectin matrix due to the loss of the fibronectin receptor while both Band 3 and ankyrin are enriched in the reticulocyte [52]. Transferrin has been found to differentially sort to the pyrenocyte membrane while glycophorin A/TER119 segregates to the reticulocyte membrane [53, 54]. Inhibition of vesicle trafficking by MiTMAB (dynamin inhibitor) prevented sorting of the transferrin receptor (CD71) towards pyrenocyte side of membrane of enucleating erythroblasts [34]. Thus, sorting of proteins and enucleation appear to be coupled in that both require vesicle trafficking. Primitive erythroblasts also differentially sort proteins, such as TER119 (reticulocyte) and α4-integrin (pyrenocyte) in a manner similar to definitive erythroblasts [55]. This difference in composition of the membranes of pyrenocyte versus reticulocyte likely assists pyrenocytes to attach to macrophages while allowing reticulocytes to move into the circulation.

Further, nuclear positioning, an essential component of enucleation (Figure 1), can be speculated to depend on protein sorting and vesicle trafficking. Interestingly, the observations by Skutelsky and Danon [31] and Ji et al. [56] about the nuclear positioning supports this notion. The former group, using fixed sections, noticed that some erythroblasts were having protrusion of the plasma membrane along with a portion of nuclei. They saw a variety of sizes of this protrusion, including in some cells the nucleus completely occupied inside the cavity. Using these pieces of fixed section observations, they constructed a model in which the nucleus at first occupies an eccentric position adjacent to the cell membrane and a cytoplasmic protrusion ensues taking along the nuclei until the protrusion completely holds the nuclei. Ji et al. observed a similar process in murine fetal liver erythroblasts using live cell imaging. These findings suggest that the visible constriction zone on the surface of enucleating erythroblast is indeed a junction region in the plasma membrane that separates pyrenocyte and reticulocyte membrane. The membrane that is destined to enclose pyrenocyte that is in close proximity to nucleus lacks actin cytoskeleton, spectrin, and other critical proteins and as a result can be visualized to balloon out without resisting the pressure exerted by the cytoskeletal activity (Figure 2).
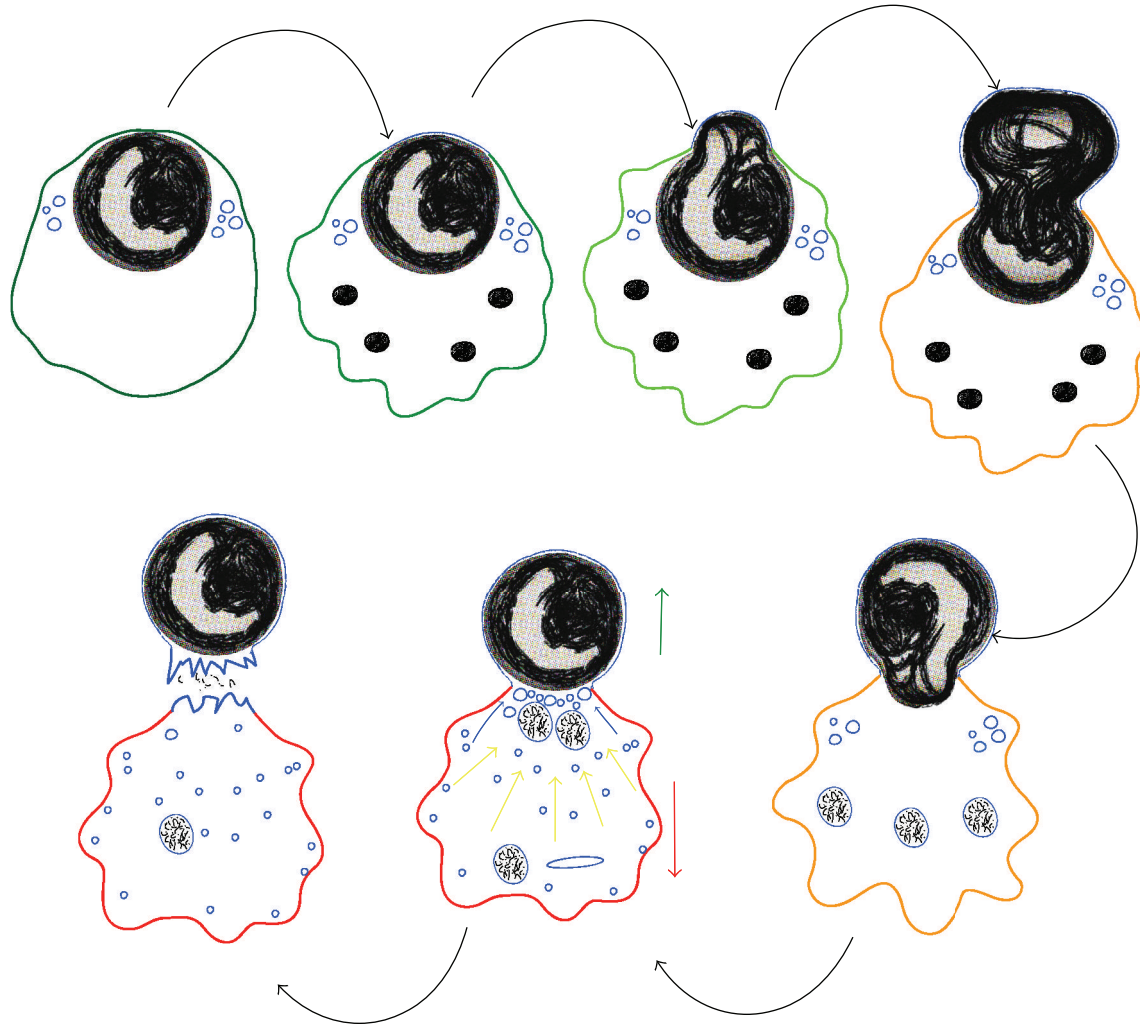
FIGURE 2: Model of enucleation. A schematic representation of the enucleation process in mammals is shown. Yellow arrows denote the direction of force applied over the nucleus by actin cytoskeleton. Blue arrows and vesicles denote the protein trafficking, which directs the proteins that are destined to reach the pyrenocyte. The blue membrane adjacent to the nucleus is the part of the pyrenocyte membrane lacking spectrin, glycophorin A, actin cytoskeleton, and other reticulocyte-specific proteins that are differentially sorted. The green arrow represents the direction of the force exerted on the pyrenocyte by a bound macrophage while the red arrow indicates the movement of the reticulocyte away from the center by lamellipodia and filopodia.

3.3. Macrophages and Enucleation. Pyrenocytes gradually start expressing phosphatidyl serine on their surface, providing an "eat me" signal for macrophages, which engulf them [23]. The engulfed nucleus is then digested in lysosome where DNase II digests the DNA within the engulfed nuclei [57]. The importance of this pathway for continued erythropoiesis is highlighted by the phenotype of DNase II knockout mice, which die in utero due to embryonic lethal anemia. When DNase II null fetal liver progenitors are transplanted into lethally irradiated recipients, the progenitors give rise to normal erythrocytes, showing that the defect is noncell autonomous, attributable to macrophages. The important role of macrophages in enucleation is also supported by the phenotype of retinoblastoma (Rb) tumor suppressor gene knockout mice. Mutant embryos exhibited a defect in enucleation, which was attributed to a lack of

suppression of Id1 (a helix-loop-helix protein) by Rb in macrophages [58]. In vivo, definitive erythropoiesis takes place within erythroblastic islands. Macrophages reside at the center of island, with erythroblasts at various differentiation stages layered around the outside [59–61]. Hanspal and Hanspal found that the interaction between erythroblasts and macrophages is needed for normal proliferation of erythroblasts as well as for enucleation [62]. This interaction is mediated by EMP (erythroblast macrophage protein), which functions to prevent apoptosis of developing erythroblasts [63]. While these studies point to an essential role for macrophages during erythropoiesis, many groups have shown that erythroblasts cultured in vitro in the absence of macrophages undergo complete differentiation including nuclear extrusion [16, 64]. Hence we can conclude that enucleation formally can occur without macrophages.

However, in vivo macrophages appear to play important functions in enucleation and erythroid homeostasis.

3.4. Autophagy and Enucleation. Another important phenomenon that takes place during enucleation is autophagy, a process by which cellular components such as organelles and protein aggregates are catabolized [65–67]. Autophagy proceeds in multiple steps. First, a double membrane develops around the cytoplasmic cargo to be degraded by autophagy. This double membrane can be derived from either the endoplasmic reticulum [68, 69] or the plasma membrane [70, 71]. Fusion of these membranes to one other sequesters the cargo to form an autophagosome, which in turn fuses with multivesicular body/late endosome/lysosome, leading to degradation of the cargo and the inner bilayer of the double-membrane. This vacuole that contains digested cytosolic contents is called autophagolysosome [72–74].

In erythroblasts, mitochondrial clearance has been shown to be accomplished through autophagy [75]. Multiple studies have shown that Nix (Bnip3L), a Bcl-2 family member, is required for mitochondrial clearance in reticulocytes and that loss of Nix leads to anemia [76, 77]. The lack of clearance has been proposed to be the result of defective entry of mitochondria into autophagosomes [77–79]. Although loss of Nix did not affect enucleation [77, 80], it is interesting to consider the dependence of autophagy on vesicle trafficking. The inhibition of vesicle trafficking blocked formation of autophagolysosomes and resulted in enucleation defects (G.K, A.W, and JDC, unpublished data). Further studies on the relationship between autophagy, vesicle trafficking, and enucleation may shed additional light on erythrocyte maturation.

3.5. Possible Roles of Actin in Enucleation. Actin has multiple roles in a cell, including cell division, migration, junction formation, chromatin remodeling, transcriptional regulation, vesicle trafficking, and cell shape regulation [81]. In an enucleating cell, actin could be involved in maintaining the shape of the cell, and/or in maintenance of polarity of the nucleus. Actin may also assist in the formation and movement of endocytic vesicles [82, 83]. Indeed, actin has been proposed to mediate the short-range movement of vesicles and may work together with myosins V and VI and members of the kinesin family [84], to direct vesicles during enucleation. Actin is regulated by Rac proteins that play a role in the formation of lamellipodia, filopodia, membrane ruffles, and cell movement [85]. Taken together, actin likely participates in erythroid maturation by polarization of nucleus, by promoting the accumulation and coalescence of vesicles/vacuoles, and by inducing migration of the reticulocyte away from the pyrenocyte.

4. Model of Enucleation

Erythroblast enucleation is a unique process that incorporates multiple aspects of cytokinesis and vesicle trafficking (Figure 2). First, one or more cellular signals initiate the process of enucleation. At least one study suggested that p38

mitogen-activated protein (MAP) kinase (MAPK) signaling is involved in late erythroid differentiation and enucleation [86]. However, if extracellular factors either secreted by the bone marrow stroma or by erythroblasts themselves play a role in engaging yet unidentified receptors to initiate an intracellular signaling cascade/s leading to the activation of signaling molecules such as the p38MAP kinase and/or the Rac-1 GTPase that may begin the enucleation process is yet to be determined. Once the process is initiated, the actin cytoskeleton polarizes the condensed nucleus, free from intermediate filament attachments, to one side of the cell. Note that this polarization need not be random, and there may well exist novel factors that determine the polarity. At this time, the region of plasma membrane in close proximity to the nucleus yields to form a small extrusion that includes a portion of the nucleus. We speculate that vesicle trafficking and other protein-sorting pathways provide additional membrane to the pyrenocyte region, allowing expansion and further extrusion of the nucleus. The subsequent formation and coalescence of U-shaped channels and vesicles that have accumulated in the region between the nucleus and incipient reticulocyte allows for separation of the reticulocyte from the pyrenocyte. In vivo, this process is likely complemented by attachment of the pyrenocyte to a nearby macrophage coupled with actin-mediated movement of the reticulocyte away from the pyrenocyte. Thus, multiple pathways, including chromatin condensation, actomyosin motors, and vesicle trafficking work in concert to ensure orchestrated terminal maturation of red cells.

5. Future Directions

Although there have been many advances in the past decade, several aspects of enucleation remain unclear. First, what are the signaling pathways that trigger enucleation in vivo and in vitro? Second, what, if any, factors determine polarity in enucleating cells? Third, what are the contributions of macrophages and how can these cells be harnessed to improve ex vivo enucleation? Finally, which motor proteins are responsible for coordinating the movement of the nucleus and cytoplasmic vesicles? It is likely that these questions will be answered in the next decade of research.

Acknowledgments

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