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REVIEW

Cellular dynamics of mammalian red blood cell production in the erythroblastic island niche

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Abstract

Red blood cells, or erythrocytes, make up approximately a quarter of all cells in the human body with over 2 billion new erythrocytes made each day in a healthy adult human. This massive cellular production system is coupled with a set of cell biological processes unique to mammals, in particular, the elimination of all organelles, and the expulsion and destruction of the condensed erythroid nucleus. Erythrocytes from birds, reptiles, amphibians and fish possess nuclei, mitochondria and other organelles: erythrocytes from mammals lack all of these intracellular components. This review will focus on the dynamic changes that take place in developing erythroid cells that are interacting with specialized macrophages in multicellular clusters termed erythroblastic islands. Proerythroblasts enter the erythroblastic niche as large cells with active nuclei, mitochondria producing heme and energy, and attach to the central macrophage via a range of adhesion molecules. Proerythroblasts then mature into erythroblasts and, following enucleation, in reticulocytes. When reticulocytes exit the erythroblastic island, they are smaller cells, without nuclei and with few mitochondria, possess some polyribosomes and have a profoundly different surface molecule phenotype. Here, we will review, step-by-step, the biophysical mechanisms that regulate the remarkable process of erythropoiesis with a particular focus on the events taking place in the erythroblastic island niche. This is presented from the biological perspective to offer insight into the elements of red blood cell development in the erythroblastic island niche which could be further explored with biophysical modelling systems.

Keywords Erythroblastic island · Mammalian erythropoiesis · Nuclear condensation

Introduction

The red blood cell (erythrocyte) is one of three mammalian cell types that are biologically functional in the absence of a nucleus or organelles. In contrast to the other anuclear cell types, lens epithelial cells and keratinocytes, which are sedentary, human erythrocytes travel over 250 km during their 110-day lifespan (Klinken 2002; Lasch et al. 2000). This distance is slightly less than the distance from Sydney to Australia's capital city Canberra or a return trip from Hong Kong to Guangzhou and back. Circulating erythrocytes make up approximately 45–55% of the 6–7 l of human blood volume (haematocrit), and have a vital role of delivering oxygen to the tissues of the body. Every microlitre of human blood contains approximately 4 to 6 billion erythrocytes. This astronomical number of erythrocytes in blood is due to a high production and maturation rate in the bone marrow with approximately 2 million red blood cells made every second. During this process, the maturing erythroid cell loses its nucleus and other organelles, leaving behind a corpuscle filled with haemoglobin to begin the 250-km-long nomadic journey.

Erythropoiesis

The process of maturation and differentiation into mature erythrocytes is termed erythropoiesis. Erythrocytes are

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derived from haematopoietic stem cells in a stepwise differentiation manner (Al-Drees et al. 2015; Yeo et al. 2018). During erythropoiesis, multiple cellular events occur including: synthesis of haemoglobin, condensation of the chromatin and nucleus and the elimination of most organelles. Erythroid progenitors expel the condensed nuclei to become reticulocytes. Reticulocytes then undergo membrane and cytoskeletal remodelling and removal of any remnant organelles before assuming a biconcave discoidal shape typical of mature erythrocytes. Alteration of any of these major cellular events has the potential to lead to pathophysiological states such as anaemia.

Stages of erythroid maturation

The colony-forming units

The first progenitors committed to the erythroid lineage are currently identified using colony-forming assays. The earliest erythroid progenitor cells are the burst-forming unit–erythroid (BFU-E). In the bone marrow, the frequency of BFU-E is approximately 4–10 cells per 10^4 nucleated cells (Gregory and Eaves 1978; Dzierzak and Philipsen 2013). The human BFU-E has been analysed at the transcriptome level. A population enriched in BFU-E activity has been isolated from human bone marrow according to expression of the fatty acid translocase/thrombospondin receptor CD36, in the absence of expression of lymphoid and myeloid cell markers (Li et al. 2014).

The next stage of erythroid differentiation is the colony-forming unit–erythroid (CFU-E). Approximately 20–60 cells per 10^4 cells in the human bone marrow are CFU-E. In contrast to BFU-E, the proliferative potential of CFU-E is limited and CFU-E cannot self-renew (Gregory and Eaves 1978; Doyonnas et al. 2005). CFU-Es are exquisitely sensitive to erythropoietin (EPO), the major hormone stimulating erythropoiesis.

The proerythroblast

The first morphologically erythroid progenitor cell which can be identified by optical light microscopy is the proerythroblast. Proerythroblasts are large mononuclear cells (human 20–25 μm; mouse 8–13 μm) with a basophilic cytoplasm (with Giemsa stain) due to a high concentration of ribonucleic acid (Filmanowicz and Gurney 1961; Bessis 1973a; Paul et al. 1973; Sasaki et al. 1982). Human proerythroblasts possess large nuclei which occupy 75–80% of the cell volume with a nuclear/cytoplasm ratio of 8:1 (Breton-Gorius and Reyes 1976). Transmission electron microscopy (TEM) revealed that the proerythroblastic nuclei have a finely stippled chromatin pattern containing one or more prominent nucleoli (Breton-Gorius and Reyes 1976). A human proerythroblast can give rise to 16 reticulocytes within 3 days (Bessis 1973). Human proerythroblasts exhibit a large prominent nucleus with euchromatin containing nucleoli when visualized by TEM. Nuclear indentations may be observed (Rifkind et al. 1964; Bessis 1973). In contrast to other haematopoietic lineages, such as the B and T lymphoid lineages, the processes regulating the development of haematopoietic stem cells (HSC) to proerythroblasts in the bone marrow are poorly defined. While it was thought that erythroid colony-forming units were more immature than proerythroblasts, TEM performed on Day 2 colony assays revealed cells resembling polychromatophilic erythroblasts, while on Day 7, colonies contain cells that resemble orthochromatophilic erythroblasts with condensed nuclei (Elste et al. 1987).

The erythroblast

Immature proerythroblasts develop into smaller basophilic erythroblasts (human 16–18 μm; mouse 6–11 μm) (Bessis 1973; Paul et al. 1973; Sasaki et al. 1982). The chromatin of the basophilic erythroblasts becomes aggregated (heterochromatin). The cytoplasm of basophilic erythroblasts exhibits strong basophilia (dark blue) with Giemsa staining due to an increased number of ribosomes synthesizing haemoglobin. Similar to proerythroblasts, basophilic erythroblasts exhibit a high nucleus/cytoplasm ratio. Basophilic erythroblasts mature into polychromatophilic erythroblasts. Polychromatophilic erythroblasts are smaller (human 12–15 μm; mouse 5–8 μm) than basophilic erythroblasts (Bessis 1973; Paul et al. 1973; Sasaki et al. 1982). The nucleus of the polychromatophilic erythroblast is more condensed than basophilic erythroblasts. The cytoplasm of the polychromatophilic erythroblast develops a pinkish tinge (hence, the term "polychromatophilia" which means "loving several colours") when treated with Giemsa stain due to the increasing level of haemoglobin protein in the cytoplasm. The nuclei of polychromatophilic erythroblasts exhibit heterochromatin clusters (condensed chromatin) adjacent to the nuclear pores. The perinuclear cisternae of polychromatophilic erythroblasts are wider in comparison to basophilic erythroblasts and proerythroblasts.

Polychromatophilic erythroblasts mature into orthochromatophilic erythroblasts (formerly known as polychromatophilic II erythroblasts). The orthochromatophilic erythroblast is the last erythroid progenitor to possess a nucleus. Orthochromatophilic erythroblasts are the smallest progenitors (human 10–15 μm; mouse 8–13 μm) compared to other nucleated erythroid progenitors and are incapable of cellular division (Bessis 1973; Breton-Gorius and Reyes 1976; Sasaki et al. 1982; Paul et al. 1973). The orthochromatophilic erythroblasts have pyknotic nuclei with large aggregates of heterochromatin in the nuclei when imaged by TEM. The cytoplasm of an orthochromatophilic erythroblast has eosinophilic appearance with Giemsa staining due to the high concentration of haemoglobin present.

The reticulocyte

Nuclear expulsion in orthochromatophilic erythroblast gives rise to reticulocyte (formerly known as pro-erythrocyte). During steady-state erythropoiesis, reticulocytes remain in the bone marrow and mature further over the next 24 to 48 h (Liu et al. 2010a). The size of the reticulocytes is approximately 8–10 μm in humans and 6–8 μm in mice (Paul et al. 1973; Liu et al. 2010a). Reticulocytes exhibit reticular (net-like) aggregates of blue material in the presence of supravital stains such as cresyl blue, hence the name reticulocytes. The cytoplasm of anuclear reticulocytes stains predominantly pink with Giemsa stain because of the high concentration of haemoglobin (Bessis 1973). Historically, nascent reticulocytes, which had just expelled their nuclei, were known as macroreticulocytes, while reticulocytes containing high concentrations of ferritin are known as normal siderocytes (Bessis 1973). Newly formed reticulocytes retain organelles such as the mitochondria and polyribosomes (Liu et al. 2010b; Rifkind 1964). Nascent reticulocytes have a greater surface area than mature reticulocytes. The expelled erythroid nucleus surrounded by a thin rim of cytoplasm is known as a pyrenocyte (McGrath et al. 2008b).

The cell membrane of the nascent reticulocyte is mechanically less stable than that of a mature erythrocyte. The membranes of immature reticulocytes undergo active exocytosis, which is not observed in normal, mature erythrocytes (Liu et al. 2010a; Yeo et al. 2016). Membrane remodelling occurs by membrane internalization followed by exocytosis of the membrane and cytoplasmic fragments (Gasko and Danon 1974). Ferritin-laden vacuoles and transferrin receptors are also removed by exocytosis in clathrin-coated pits (Liu et al. 2010a; Malleret et al. 2015). Reticulocytes undergo a further maturation in the bone marrow for up to 48 h before becoming circulating erythrocytes (Bessis 1973). Reticulocytes then leave the bone marrow and circulate for 24–48 h. In normal human blood, reticulocytes constitute approximately 1 to 2% of the total erythrocyte count (Bessis 1973) (Liu et al. 2010a). Reticulocytes enter into the circulation via diapedesis by extending pseudopods through the wall of marrow capillaries (Bessis 1973). In normal human blood, reticulocytes constitute approximately 1 to 2% of the total erythrocyte count (Liu et al. 2010b; Bessis 1973).

The erythrocyte

Erythrocytes (also known as discocytes) circulate in the peripheral circulation for approximately 110 days (varying from 70 to 140 days in a healthy individual) (Shemin and Rittenberg 1946). Circulating erythrocytes facilitate gaseous exchange by transporting oxygen via haemoglobin. Human erythrocytes are small biconcave discs, which appear dark and refractile by phase contrast microscopy and stain “bright” pink/red with Giemsa stain (Bessis 1955; Bessis 1973). Erythrocytes vary in size across mammalian species. The average size of a human erythrocyte is approximately 6–8 μm (Bessis 1973) while a mouse erythrocyte measures 4–6 μm (Filmanowicz and Gurney 1961; O'Connell et al. 2015; Green 1966). When visualized by TEM, erythrocytes are electron opaque structures filled with haemoglobin containing no cellular organelles (Bessis, 1973; Bessis and Weed 1973; Polliack 1981).

Erythroblastic islands

Erythroid cells mature from proerythroblasts to reticulocytes within specific niches in the erythropoietic organs such as the foetal liver, bone marrow and spleen (Chasis and Mohandas 2008; Manwani and Bieker 2008). These niches are composed of a central macrophage surrounded by and interacting with developing erythroid cells (Bessis 1958) and are termed erythroblastic islands (EBI). EBI can be readily isolated from the foetal liver or bone marrow by gentle dissociation of the haematopoietic tissues, sedimentation and brief culture on glass coverslips (Bessis 1958; Yeo et al. 2018). The central macrophage of the erythroblastic island (herein termed EBI-CM), with the developing erythroid cells attached, can adhere to the glass coverslip within 1 h of culture (Yeo et al. 2018). The foetal liver and bone marrow are both sources of EBIs. In some species, EBIs have also been isolated from the spleen. The species from which EBIs have been isolated include: laboratory mouse and rat strains, guinea pigs, rhesus monkeys, humans (with pathological conditions) and the carnivorous marsupial fat-tailed dunnart (Yeo et al. 2016). We, and others, have described the structure and possible functions of EBI (An and Mohandas 2011; Yeo et al. 2019). Here, we focus on the biophysical events taking place within this niche (Table 1).

The macrophage compartment of erythroblastic islands

What is the nature of the macrophage compartment of erythroblastic islands? EBI-CM from the bone marrow of laboratory mice have been reported to express: Mac1, the haemoglobin/haptoglobin receptor CD163, CD169/Sialoadhesin, vascular cell adhesion molecule-1 (VCAM1), F4/80 antigen, the phosphatidylserine receptor Tim4, α_V integrin chain and the Forssman glycoantigen (Sadahira et al. 1991; Lee 2006; Isern et al. 2008; Chow et al. 2013; Fraser et al. 2015). Using multispectral imaging flow cytometry, CD169, F4/80 and VCAM1 were found to be variably expressed by EBI-CM whereas CD11b was not expressed by EBI-CM (Seu et al. 2017). These data suggest that EBI-CMs are a heterogenous population and that EBI-CM may be a unique macrophage population.

Jacobsen and colleagues have used granulocyte-colony stimulating factor (G-CSF) or Flt3-ligand treatment to

Table 1 Glossary of frequently used terms

Term	Meaning
Haematopoeisis	The constant production of blood cells
Erythropoiesis	The ongoing production of red blood cells also known as erythrocytes
Bone marrow	The spongy tissue inside bones which serves as the primary site of haematopoiesis in adult humans
	Abbreviated to BM in this review
Erythroblast	Precursor cell of the mature red blood cell, the erythrocyte
Macrophage	Functionally mature phagocytic cells
Erythroblastic island	Multicellular cluster of developing red blood cell precursors-erythroblast-surrounding a mature, functional macrophage
	Abbreviated to EBI in this review
Erythroblastic island central macrophage	The macrophage surrounded by developing erythroid cells in the EBI. Engulfs and destroys expelled erythroid nuclei
	Abbreviated to EBI-CM in this review
Nuclear condensation	The condensation of the erythroid nucleus to a fraction of its original size
Enucleation	The process of removing the erythroid nucleus from the orthochromatophilic erythroblast
Pyrenocyte	The expelled erythroid nucleus encapsulated in erythroid cell membrane with a perdurant amount of cytoplasm carrying haemoglobin

interfere with the bone marrow niche Ly6G (Jacobsen et al. 2014; Jacobsen et al. 2016). These treatments result in a transient reduction in erythropoiesis and alterations in macrophage populations. This group have reported that EBI macrophages express F4/80, VCAM1, CD169 ER-HR3 and Ly6G (Jacobsen et al. 2014; Jacobsen et al. 2016). While there are clear differences in this macrophage population following G-CSF or Flt3-ligand treatment, the authors have not sorted this specific population and assessed EBI formation in comparison to other macrophage populations from the marrow. EBI-CM in the *Csfr1-GFP* transgenic mouse strain expresses GFP (Sasmono et al. 2003). EBI-CM also expresses the cytoplasmic enzyme, heme oxygenase-1 abundantly (Fraser et al. 2015). Rat EBI-CM expresses the haemoglobin-haptoglobin scavenger receptor CD163 (Fabriek et al. 2007).

We have identified two morphologically distinct classes of EBI which can be isolated from the mouse, guinea pig and dunnart marrow (Yeo et al. 2016). Flat and domed EBI are distinct in terms of the central macrophage morphology (smaller, raised, domed macrophages vs. larger, flatter macrophages). These EBIs also differ in type and frequency of erythroid cell attached to the macrophage, suggesting that these are functionally different niches. It is not yet known if these macrophages are distinct lineages of macrophages or if they are part of a continuum where one form becomes another depending on the needs of the erythroid cells interacting with the macrophage. The differences between flat and domed EBI are shown in Fig. 1.

Are the central macrophages of erythroblastic islands (EBI-CM) a unique macrophage subtype? These macrophages have unique properties compared to mature macrophages in other organs. For example, EBI-CM does not engulf and destroy the entire erythroid cell, as splenic red pulp macrophages do. The phagocytic ability of EBI-CM appears to be restricted to pyrenocytes under normal conditions. During haemophagocytic histiocytosis, the phagocytic behaviour of bone marrow macrophages can change with the macrophages engulfing and destroying entire haematopoietic cells. EBIs may play a role in haematological disease. Depletion of EBI-CM in mouse models of polycythaemia vera (a myelodysplastic syndrome producing too many erythrocytes) and β-thalassaemia led to an improvement in the health of the animals (Chow et al. 2013; Ramos et al. 2013).

Step-by-step production of erythroid cells in the erythroblastic niche

How does the bone marrow produce over 2 billion new erythrocytes each day? The niche responsible for this massive cellular production is the erythroblastic island. Proerythroblasts attach to the EBI-CM as large nucleated cells with active mitochondria, endoplasmic reticula and Golgi apparati, and later detach from the EBI as anuclear reticulocytes harbouring vast amounts of haemoglobin and a small number of mitochondria while decorated with only a fraction of the adhesion molecules compared to their progenitor population. Here, we will detail the step-by-step processes a proerythroblast must proceed through to become a reticulocyte with a particular focus on the biophysical processes involved in erythroid maturation in the erythroblastic niche.

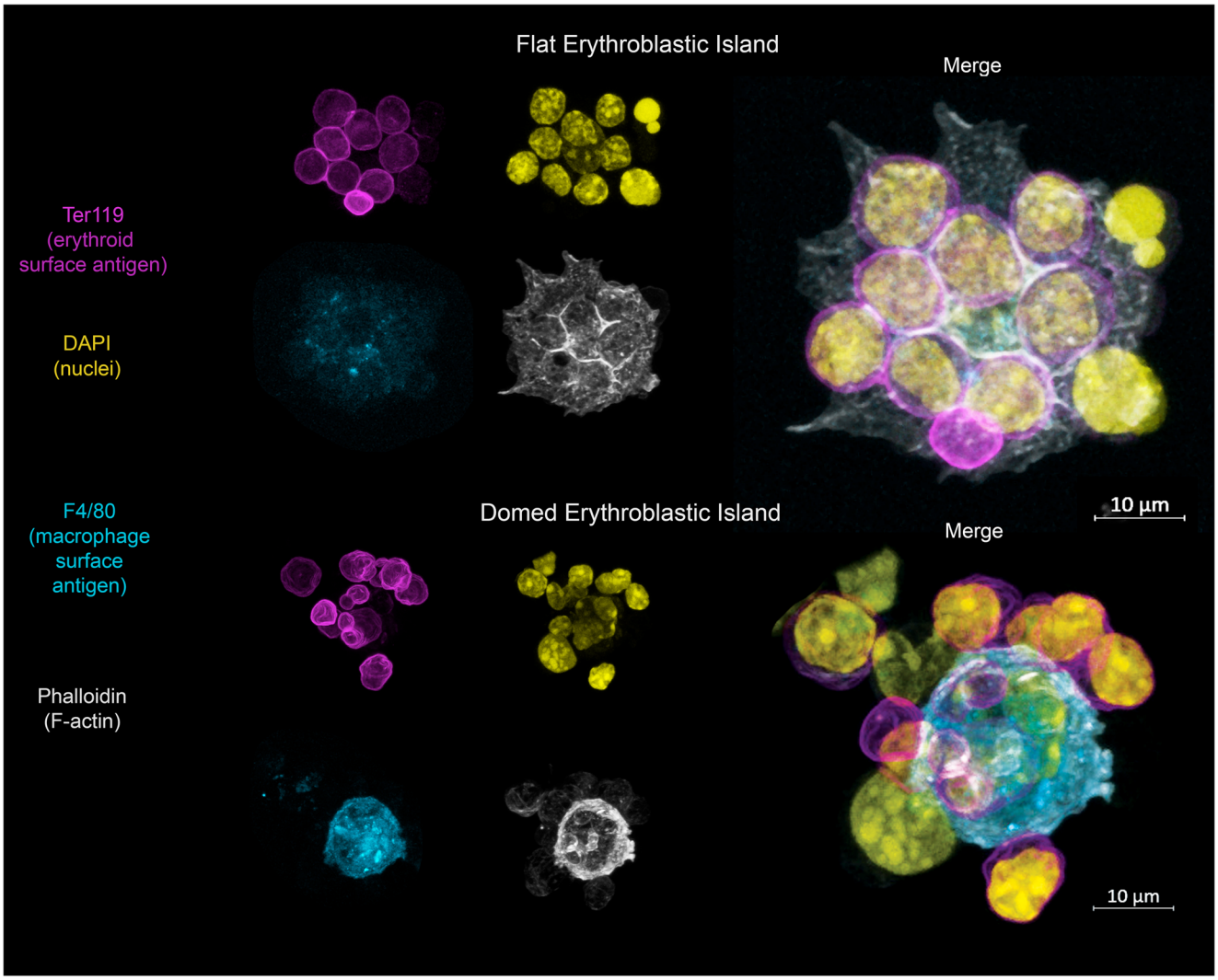

Fig. 1 Flat and domed erythroblastic islands isolated from the mouse bone marrow. EBI were isolated from healthy adult wild-type mouse femoral marrow and immunostained with Ter119 antibody highlighting the erythroid population (magenta) and F4/80 antibody (cyan) to identify the central macrophage. DAPI counterstains the nucleus (yellow) and Phalloidin binds to F-actin (grey). The differences in macrophage morphology are highlighted by the distinct F-actin profiles. These images are maximal projection images of z-stack confocal images. Individual fluorescent channels are shown on the left hand panel; larger merged images are shown in the right panel

Step 1: Erythroblast attachment to macrophages in EBI

Erythroblast attachment to EBI central macrophages

Erythroid progenitors interact with EBI-CM from as early as the proerythroblast stage. As the current set of markers that delineate erythroid differentiation stage are primarily markers related to mature stages, it is challenging to determine the precise stage at which proerythroblasts attach to EBI-CM. Using scanning electron microscopy, we have previously dissected the differentiation stages according to size, presence or absence of microvilli on the cell surface and expression of the transferrin receptor CD71 (Yeo et al. 2016). Proerythroblasts are the largest erythroid cell attaching to EBI-CM. The surface of proerythroblasts is characterized by the presence of ridge-like protrusions or a form of microvilli (Yeo et al. 2016). A similar morphology was observed using SEM and TEM in Dexter cultures of maturing blood cells and in colonies from the spleens from phlebotomised mice. As the proerythroblasts mature into erythroblasts, the surface morphology and antigen expression domains shift. We have observed CD71 move to the edge of the ridge-like microvilli and Ter-119 appear in a similar expression profile (Yeo et al. 2018).

A number of adhesion molecules are known to play important roles in erythroblast-macrophage interactions and to establish the integrity of the EBI. Integrins $\alpha 4\beta 1$ are expressed by erythroblasts to facilitate attachment to VCAM-1 expressed by EBI-CM. Erythroblasts also express $\alpha 5\beta 1$ though expression of the cognate ligands of this integrin (fibronectin and vitronectin) by

EBI-CM is yet to be confirmed. The number of α4β1 integrin-expressing erythroblasts and VCAM-1-expressing macrophages forming EBIs is reduced in the spleens of mice that are either on a selenium-deficient diet, or genetically deficient in selenoprotein synthesis (Liao et al. 2018). The precise mechanisms behind this are currently unclear (Liao et al. 2018). The tetraspanins CD81 and CD82 are expressed by maturing human erythroblasts in culture, and co-immunoprecipitate with α4β1 integrins (Spring et al. 2013). The authors of this work suggest the CD81 and CD82 help to organize α4β1, improving binding of the erythroblast to VCAM1 expressed by the EBI-CM (Spring et al. 2013).

Intercellular adhesion molecule-4 (ICAM4) tethers erythroblasts to α_V integrin on EBI-CM (Lee 2006). CD44 is highly expressed by mouse erythroblasts and by human erythroblasts and indeed erythrocytes, which variant forms of CD44 give rise to the Indian blood group system (Chen et al. 2009). CD44 can interact with hyaluronic acid as well as other ligands including osteopontin, matrix metalloproteinases and some forms of collagen. None of these ligands have been formally demonstrated to be expressed by EBI-CM.

In an elegant study with surprising outcomes, Anselmo and colleagues found that EphB1 on erythroblasts is clustered by dystroglycan, a component of the myocyte cytoskeleton, better known as a gene frequently mutated in muscular dystrophy (Anselmo et al. 2016). Agrin, present on erythroblasts, clusters dystroglycan and EphB1 which then transduces a signal into the erythroblast. Mice lacking functional agrin protein die soon after birth and show a high degree of anaemia and altered erythroblastic island structure. EphB1 activation in erythroblasts led to increased expression of α5β1 integrin but not $\alpha_v\beta3$ integrin. The haemoglobin/haptoglobin receptor CD163 is expressed by rat bone marrow macrophages and is an erythroblast receptor in EBI (Fabriek et al. 2007). The domain of CD163 that binds to erythroblasts is distinct from the haemoglobin/haptoglobin domain though the erythroblast ligand for CD163 has yet to be determined (Fabriek et al. 2007).

Erythroblast macrophage protein (Emp), encoded by the macrophage erythroblast attacher gene (Maea), regulates EBI integrity. *Maea*-deficient erythroid cells show a severe defect in enucleation and a profoundly altered EBI morphology. In a very recent study, Wei and colleagues have conditionally deleted the *Maea* gene from macrophages in situ and observed that macrophage-expressed Maea/EMP is the crucial form in regulating EBI integrity (Wei et al. 2019). However, contrary to previous studies, deletion of the *Maea* gene in the erythroid compartment did not have a significant impact on erythropoiesis or enucleation (Soni et al. 2006; Soni et al. 2008; Wei et al. 2019).

Downstream events following erythroblast-macrophage interaction

Following attachment of proerythroblasts to EBI-CM, signalling via adhesion molecules such as integrins, EphB1 and Maea/EMP leads to remodelling of the actin cytoskeleton in both the erythroid cell and the macrophage. Maea/EMP protein expression is considered to have multiple roles including cell-cell attachment as well as actin and nuclear matrix-binding activity (Soni et al. 2006; Soni et al. 2007). Palladin also interacts with the actin cytoskeleton, and palladin-deficient embryos show a reduction in enucleation, loss of EBI formation and die in utero from anaemia (Liu et al. 2007). The pointed end of F-actin is capped by tropomyosin and tropomodulin proteins. Tropomodulin 1 regulates F-actin function during enucleation (Nowak et al. 2017). Tropomodulin 3 is expressed by both erythroblasts and macrophages in EBIs, and mutation of Tmod3 (the gene encoding tropomodulin 3) results in severe anaemia, defects in EBI structure and formation and embryonic lethality (Sui et al. 2014). Mutation of the serine threonine kinase *Stk40* gene leads to severe anaemia (Wang et al. 2017). Wang and colleagues observed the *Stk40* deletion in erythroblasts but not macrophages led to disruption of EBI integrity (Wang et al. 2017). These results indicate an erythroid-specific response to erythroblast-macrophage attachment regulated by Stk40. Rac1 and Rac2, Rho-specific kinases, regulate actin dynamics within erythroblasts, though their function in EBI-CM and contribution to the integrity of EBI is yet to be reported (Kalfa et al. 2010).

Genetic regulation of EBI formation

Several transcriptional regulators have been reported to play roles in the establishment and maintenance of EBI. The proto-oncogene c-Maf regulates expression of the macrophage marker F4/80, as well as VCAM-1, a ligand of erythroblastic α4β1 integrin (Nakamura et al. 2009; Kusakabe et al. 2011). While there is a greatly increased frequency of nucleated erythrocytes from mice lacking both copies of *Mafc*, the gene that encodes c-Maf protein, erythropoiesis is relatively normal (Kusakabe et al. 2011). *Mafc*-deficient foetal liver macrophages, however, showed reduced levels of VCAM-1 expression as well as decreased expression of *Maea* (encoding Emp), and the genes encoding CSFr1, the mannose receptor and L-selectin (Kusakabe et al. 2011). Kruppel-like factor-1 (Klf1), also known as EKLF, is a transcriptional regulator expressed highly in developing erythroblasts and also in EBI-CM (Xue et al. 2014). Klf1 regulates expression of adhesion molecules on erythroid populations that are required for EBI stabilization such as α4β1 and α5β1 integrins as well as CD44 (Isern et al. 2010). Within the erythroid compartment, Klf1 directly regulates expression of ICAM-4, a requisite erythroid adhesion molecule which was described above as interacting with $\alpha_v\beta3$ integrin on EBI-CM. EBI integrity is dependent upon the expression of Klf1 (Xue et al. 2014). *Klf1* also regulates the expression of DNAse II, an enzyme essential in degrading erythroid nuclei engulfed by macrophages (Porcu et al. 2011).

Step 2: Nuclear rearrangement and condensation

Genome packaging

During terminal erythroid cell differentiation, the nuclear size decreases dramatically (McGrath et al. 2017). Morphometric analyses of TEM images of murine neonatal spleen cells showed that the nuclear volume of erythroid cells varied from 230 to 33 μm^3, suggesting significant nuclear compaction during erythroid development (Sasaki et al. 1982). The nuclear diameter of erythroid progenitor cells isolated from peripheral primitive erythroid cells decreases by threefold from E9.5 to E14.5 (Fraser et al. 2007). A similar shrinkage of nuclear size takes place during the in vitro differentiation of erythroid progenitor cells (Ji et al. 2008). The decrease in nuclear size is accompanied by chromatin condensation. Using Friend virus-infected murine spleen erythroblasts as a model, Popova et al. (2009) reported that, while nucleosomal composition was unchanged, there was a significant increase in histone H3 dimethylation and a reduction of histone H4 acetylation during terminal erythroid differentiation (Popova et al. 2009). Similarly, a decrease of acetylation on H3 K9 and H4 K5, K8, K12 and K16 in erythroid progenitor cells after 48 h of erythropoietin stimulation (Jayapal et al. 2010). The role of H4 in the higher order folding of chromatin, especially inter-nucleosome interactions, is well established (Dorigo et al. 2003). H4K16Ac significantly reduces inter-nucleosome stacking and chromatin folding possibly through the disruption of the stacking efficiency and of the electrostatic interactions of H4 to the acidic patch of the neighbouring nucleosomes (Liu et al. 2011; Winogradoff et al. 2015; Zhang et al. 2017). Hence, deacetylation of H4 may reverse this disruption and promote chromatin condensation.

During mitosis, the global phosphorylation of H3S10 by Aurora B kinase recruits histone deacetylases (HDAC) to the chromatin and triggers chromatin condensation (Wilkins et al. 2014; Hendzel et al. (1997). During terminal erythroid differentiation, however, class I HDACs are recruited to the chromatin by condensin II subunit mCAP-G2 (Xu et al. 2006). The functional role of histone deacetylation in nuclear condensation in erythoid differentiation is validated by a number of studies. HDAC5 is significantly upregulated throughout erythroid differentiation, accompanied by a subtle increase of HDAC6 during the initial phase of differentiation. Treatment with histone deacetylase inhibitors blocked both chromatin condensation and nuclear extrusion (Fujieda et al. 2005; Popova et al. 2009; Ji et al. 2010; Wölwer et al. 2015). RNAi-mediated knockdown of HDAC1 in human haematopoietic progenitors impairs erythroid differentiation (Wada et al. 2009). However, RNAi knockdown of histone deacetylase 2, but not of the other Class I histone deacetylases, phenotypically mimicked the effect of trichostatin A or valproic acid treatment, causing significant inhibition of chromatin condensation and enucleation in vitro (Ji et al. 2010). Disruption of Class II histone deacetylase HDAC6 by inhibition or by gene knockout impairs globin gene transcription and enucleation (Li et al. 2017). HDAC6 specifically interacts with serine 2 phosphorylated Pol II and is recruited mainly at the transcribed region of β-globin, correlating with RNA Pol II recruitment. These results suggest that HDAC6 promotes erythroblast differentiation through the regulation of transcription elongation. Terminal erythroid differentiation is blocked by ectopic Myc expression which suppresses histone deacetylation by inducing Gcn5, a histone acetyltransferase (Jayapal et al. 2010). MiR-191 targets Riok3 and Mxi1, two erythroid-enriched and developmentally upregulated genes. Riok3 and Mxi1 directly, or indirectly through c-Myc, negatively regulate Gcn5. Overexpression of miR-191, or knockdown of Mxi1 or Riok3, also blocks nuclear condensation and enucleation (Zhang et al. 2011). Interestingly, the chemical inhibition of HDACs induces the expression of foetal globin (Shearstone et al. 2016). The role of histone methylation in erythroid differentiation is less well understood. Both SetD8 and SetD are essential for erythroid development, through the epigenetic silencing of GATA2 and globin genes (DeVilbiss et al. 2015; Li et al. 2016).

Global histone remodelling, especially H4 deacetylation, is therefore likely to be important for chromatin condensation during the final stage of erythroid differentiation. The exchange between chromatin-bound and free histone H1 slows as murine erythroleukemia (MEL) cells differentiate, consistent with a reduction in histone dynamics during chromatin condensation (Yellajoshyula and Brown 2006; Martin and Cardoso 2009; Llères et al. 2009). This decrease in histone dynamics is associated with H1 dephosphorylation. Ectopic expression of a H1 mutant that mimics hyperphosphorylation blocks erythroid differentiation (Yellajoshyula and Brown 2006).

DNA methylation

Global DNA demethylation has been observed in mouse erythroid differentiation, making erythropoiesis one of the only three developmental processes associated with general DNA demethylation in mammalian cells (Shearstone et al. 2011; Yu et al. 2013). Compared to the two cell types that exhibit global DNA demethylation (primordial germ cells and the male pronucleus of the zygote), erythroid DNA demethylation is incomplete. Methylation level decreases from 61% in erythroid progenitors to 42% in post-polychromatophilic stage. Contrary to the conventional role of DNA methylation with transcriptional repression, demethylation in erythroid differentiation has no observable effect on transcription activities (Curradi et al. 2002). Ten-eleven translocation (TET2), a factor that demethylates DNA by oxidizing 5-methylcytosine (5mC) to 5-hydroxy-methylcytosine, is frequently mutated

in myelodysplastic syndromes (MDS), a group of pre-malignant hyperplastic conditions characterized by erythropoietic defects (Langemeijer et al. 2009). *TET2* knockdown led to hyper-proliferation and impaired differentiation of erythroid progenitors, RNAi-mediated knockdown of the expression of TET3 in human $CD34^+$ cells impairs nuclear condensation and extrusion (Yan et al. 2017). However, the functional significance of these observations is not clear, as the knockdown of *TET2* and *TET3* does not affect DNA methylation levels. As erythroid demethylation requires cell cycling, this phenotype is more likely due to the lack of methylation after new rounds of DNA replication (Shearstone et al. 2011). This is consistent with the observation that DNA methyltransferase 1 activity is not essential for the differentiation of the myeloerythroid lineage (Bröske et al. 2009).

Higher order chromatin organization and regulation of gene expression

Analyses of histone modification states at the β-globin locus control region (LCR) were crucial in establishing the role of histone modifications in transcriptional regulation such as the effect of H3K9 methylation on elongation at the LCR11 and histone hyperacetylation on high-level β-globin expression (Fromm and Bulger 2009). In proliferating cells expressing *Myb*, enhancers within the Myb-Hbs1l intergenic region are found in active chromatin hubs (ACH) containing the *Myb* promoter and first intron (Stadhouders et al. 2012). ACH is destabilized by a loss of chromatin looping in that region in differentiated erythroid cells and *Myb* expression is silenced. More recently, ChIP-seq has provided a global view of the dynamics of promoter and enhancer usage during erythroid differentiation (Su et al. 2013; Lara-Astiaso et al. 2014).

Nonhistone proteins

E2F-2, a retinoblastoma (Rb)-regulated transcription factor, is highly expressed in terminal erythroid maturation and regulates chromatin condensation and enucleation (Swartz et al. 2017). Hyper-phosphorylation of Rb, caused by the deregulation of Cyclin E, stalls terminal erythroid differentiation by activating p53 and inducing reactive oxygen species accumulation (Xu et al. 2014). However, deletion of E2F-2 abolished nuclear condensation and enucleation. E2F-2 mediates an effect on chromatin condensation in erythroid cells through the expression of citron Rho-interacting kinase (CRIK) (Swartz et al. 2017). Broad-spectrum kinase inhibitor treatment leads to nuclear condensation defects during erythropoiesis. CDK9 inhibitors suppress enucleation while inhibitors of CDK2 and CDK6 trigger erythroid differentiation (Matushansky et al. 2000; Wölwer et al. 2015).

The nuclear envelope

The nuclear envelope also undergoes profound structural changes during erythroid differentiation. The nuclear pore component Nup62 coalesces from an even distribution in early erythroid progenitors to a number of large aggregates on the nuclear envelope in late erythroid cells (Krauss et al. 2005; Zhao et al. 2014; Zhao et al. 2016). This may reflect a breakdown and redistribution of the components of the nuclear pore complex or a rearrangement of intact nuclear pores. Freeze-etching electron microscopic studies estimated that about 9–11 nuclear pores per square micrometre of nuclear envelope surface in cycling mammalian cells (Maul and Deaven 1977).

Extruded mouse erythroid nuclei have a much lower protein content compared to progenitor cells (Hattangadi et al. 2014). Nuclear proteins, including histones, are lost through nuclear export through Exportin 7 (XPO7), a factor essential to nuclear condensation and extrusion (Hattangadi et al. 2014). Expression of XPO7 is essential for erythroid differentiation and the overexpression of miR181, which targets XPO7, inhibits enucleation (Figueroa et al. 2018). Nuclear openings, sometimes up to the size of 40% of the entire nucleus, appear transiently during erythroid differentiation (Zhao et al. 2014; Zhao et al. 2016). Caspase-3 is required for the formation of these opening, possibly through lamin B cleavage (Carlile et al. 2004). The disruption of nuclear lamin and caspase-mediated degradation of lamin B reported in that study contradict with earlier observations and suggests the occurrence of a novel process during erythroid differentiation (Krauss et al. 2005). However, it is not clear how these openings affect the nuclear functions of these erythroid precursors, and how the openings are resealed in subsequent stages. The integrity of the nuclear lamina is important for the regulation of erythroid differentiation, as the disruption of the lamin-LAP2α complex leads to hyper-proliferation of erythroid progenitor cells (Naetar et al. 2008).

Global changes in chromatin accessibility have never been observed during mammalian erythroid differentiation when assayed by DNase sensitivity, DNase-seq or atac-seq (Popova et al. 2009) (Lara-Astiaso et al. 2014) (Corces et al. 2016). All histones, with the exception of H2AZ, are partially released into the cytoplasm and degraded by the proteasome pathway (Zhao et al. 2016). The appearance of histones in the cytoplasm is consistent with a recent proteomic study which detected a large amount of H4 in reticulocytes (Gautier et al., 2016). The stoichiometric balance of histone subtypes may be important for erythropoiesis, as the null mutation of H2AX inhibits enucleation (Zhao et al. 2016).

The nucleolus

The nucleolus is not prominent in late erythroid cells when assessed by light microscopy. Indeed, nucleoli are almost

invisible beyond the basophilic erythroblast stage. Classical ultrastructural studies by Karel Smetana (1984) and Olga Zatsepina (1988) indicated that during mouse erythroid differentiation there is a progressive reduction of nucleolar size and a loss of dense fibrillar regions and granular centres (Smetana and Likovský 1984; Zatsepina et al. 1988). The number of nucleolar organizer regions (AgNORs), the only visible nucleolar substructure in polychromatic erythroblasts and normoblasts, is significantly reduced. These observations are confirmed by nucleophosmin immunostaining of mouse erythroblasts (Hayashi et al. 2014). The breakdown of nucleoli corresponds to the decrease of ribosomal RNA production, as shown by metabolic labelling and pre-rRNA detection (Fraser and Curtis 1987; Jarzebowski et al. 2018). In spite of the silencing of rRNA synthesis, the global translational efficiency remains largely unchanged during erythroid differentiation (Alvarez-Dominguez et al. 2017). More studies are needed to reconcile the maintenance of high translational output in the absence of rRNA biogenesis, as the half-life of rRNA in mammalian erythroid progenitors is only about 30 min (Hunt 1976).

A specific ribosomal rescue pathway has recently been discovered in reticulocytes, via the downregulation of ribosome recycling factor ABCE1 and induction of the ribosome rescue factor PELO during erythroid differentiation (Mills et al. 2016). Defects in the translation machinery can severely affect erythropoiesis, as exemplified by Diamond-Blackfan anaemia, a genetic disease that involves the mutations or deletions in ribosome protein genes (Flygare and Karlsson 2007). Reduced translational efficiency in Diamond-Blackfan anaemia compromises the production of important erythropoietic regulators such as GATA1 (Horos et al. 2012) (Ludwig et al. 2014). The imbalance of ribosomal protein production also causes nucleolar stress accompanied by reorganization of the nucleolar structure, p53 activation and cell cycle arrest (Boulon et al. 2010).

Transcription and splicing machinery

RNA polymerase II (RNAPII) transcription occurs in specialized nuclear structures known as transcription factories (Schoenfelder et al. 2010; Rieder et al. 2012). Transcription factories in mouse foetal liver erythroblasts are an order of magnitude larger than those observed in HeLa cells (Eskiw and Fraser 2011). The largest transcription factories include those which contain KLF1. These KLF1-containing transcription factories also contain PML oncogene and splicing factor SC35 suggesting a higher-order coordination of nuclear architecture in the regulating erythroid gene expression. KLF1 is mainly cytoplasmic in the early erythroid progenitor and is selectively targeted into these nuclear foci during differentiation (Shyu et al. 2007). Klf1 protein shuttles in and out of the nucleus suggesting a role for Klf1 in the cytoplasm as well as playing a role in the nucleus (Quadrini et al. 2008). GATA1 is recruited into the same structures by PML4, a process important to enhance the trans-acting activities of GATA1 (Wu et al. 2014). It is likely that these nuclear domains are specialized "organelles" for the transcription of erythroid-specific genes.

CCCTC-binding factor (CTCF), a transcription factor responsible for organizing the higher-order folding of genome in the nucleus, is redistributed from the nucleoplasm to the nucleolus during erythroid differentiation (Kim et al. 2015; Torrano et al. 2006). Splicing snRNPs cluster from nuclear speckles to large aggregates during erythroid differentiation (Antoniou et al. 1993; Krauss et al. 2005). These changes are similar to those observed in cells after transcription inhibition (Lamond and Spector 2003). Interestingly, splicing snRNPs and Cajal body do not form nucleolar caps, another hallmark of transcription inhibition which are observed during the enucleation of lens epithelial cells (Shav-Tal et al. 2005; Dahm et al. 1998). This suggests that the sorting of nuclear proteins may be distinct for each enucleating cell type. During terminal erythroid differentiation, splicing snRNPs are localized to a membrane bound vesicle associated to the nucleus just prior to enucleation (Antoniou et al. 1993). During apoptosis, ribonucleoproteins have been observed to be pushed to one side of the nucleus, segregated from the condensed chromatin (Biggiogera et al. 2004). It is not known whether these membrane vesicles represent a final step of this segregation nor is it clear how these vesicles are structurally and functionally related to the perinuclear vacuoles observed in enucleating erythroid cells (Keerthivasan et al. 2010).

Step 3: Programmed destruction of organelles

During erythropoiesis, the loss of erythroid organelles occurs even after the expulsion of the erythroid nuclei. It was largely assumed that most organelles are degraded by autophagy. This section aims to provide morphological information of major cellular organelles outside of the nucleus.

Mitochondria

Mitochondria in erythroblasts, as well as producing energy through oxidative phosphorylation, perform a uniquely erythroid role in synthesizing haemoglobin. Previously, we have shown that mitochondrial ROS is highly dynamic in developing erythroid cell types (Kaur et al. 2015; Kaur et al. 2016). Mitochondrial biogenesis is elevated in immature stages of erythropoiesis, however, erythrocytes must be devoid of mitochondria for healthy function. Despite the importance of mitochondria and mitophagy in erythropoiesis, our understanding of how erythroid mitochondrial function is regulated in the EBI niche is unclear. Autophagy of mitochondria, or mitophagy, is thought to primarily occur following enucleation (Liu et al. 2010b). Deficiency in factors essential in

mitophagy results in anaemia in mutant mouse models. Sphingosine kinase 1 (Sphk1) regulates the mitophagic factors Nix/Bnip31 and Pink1 (Yang et al. 2019). *Sphk1*-deficiency leads to disruption of Nix and Pink1 expression (Yang et al. 2019). *Nix*-deficient mice exhibit circulating reticulocytes with significant amounts of mitochondria, which in turns leads to an anaemic state in these mutant mice (Schweers et al. 2007). Mice lacking the autophagy-related gene *Atg7* present with a broad range of erythroid pathologies due to their inability to completely eliminate mitochondria from their circulating erythrocytes (Zhang et al. 2009b; Mortensen et al. 2010). These pathological conditions include: erythroid maturation blockade, anaemia, decreased lifespan of erythrocytes and splenomegaly or enlarged spleen resulting from the increased need to remove damaged erythrocytes from the circulation (Mortensen et al. 2010; Mortensen and Simon 2010). While approximately 2% of wild-type mice have some erythrocytes bearing mitochondria, in conditionally *Atg7*-deleted mice, this frequency climbs to up to 40% of erythrocytes (Mortensen et al. 2010). Mutation of the mitochondrial DNA polymerase *Polg* disconnects the link between iron uptake and mitochondrial clearance, leading to erythrocytes circulating with mitochondria and significant haematological problems (Ahlqvist et al. 2015). Removal of mitochondria from erythrocytes is therefore a requisite process for the production of physiologically appropriate mature erythrocytes in mammals.

The endoplasmic reticulum (ER) and Golgi apparatus

Unlike granulocytic progenitors, erythroid progenitors do not have a well-developed endoplasmic reticulum (Pease 1956). "Rare strands" of rough ER (up to 2.5 μm in length) can be observed in the cytoplasm of human proerythroblasts (Orlic et al. 1965; Breton-Gorius and Reyes 1976; Kamiyama 1971). Grasso and colleagues had reported traces of rough ER in human polychromatophilic erythroblasts (Grasso et al. 1978). Rare smooth endoplasmic reticula in immature human erythroblasts are in small vesicular forms (Kamiyama 1971). Along with the Golgi apparatus, there is no known report of the endoplasmic reticulum after the proerythroblast in the erythroid maturation series. This is puzzling as the mature erythrocyte expresses a broad range of heavily glycosylated surface molecules including glycophorins A, B and C, as various glycosylated blood group antigens. The elimination of the endoplasmic reticulum is presumably due to an *Atg-7*-independent autophagy or non-autophagic processes during erythropoiesis (Mortensen et al. 2010; Moras et al. 2017).

Well-developed Golgi apparati can be observed in the cytoplasm of human proerythroblasts (Orlic et al. 1965; Breton-Gorius and Reyes 1976; Kamiyama 1971). The human proerythroblastic Golgi apparatus, which is either "small or medium" in size, surrounds the centrioles in the human proerythroblast (Orlic et al. 1965; Breton-Gorius and Reyes 1976; Kamiyama 1971). Grasso and colleagues observed the presence of Golgi in a human polychromatophilic erythroblast (Grasso et al. 1978). In rats, stacks of Golgi cristae were observed in orthochromatophilic erythroblasts (Heynen and Verwilghen 1982). It is unknown if the Golgi undergo autophagy.

Ribosomes

Multiple ribosomes can aggregate into rosettes (on a strand of mRNA) forming polyribosomes found in the cytoplasm of human proerythroblasts and basophilic erythroblasts (Bessis 1973; Rifkind 1964). The frequency of polyribosomes decreases as erythroid progenitors mature into polychromatophilic erythroblasts, and further decreases in orthochromatophilic erythroblasts. The polyribosomes disaggregate into mono-ribosomes in anuclear reticulocytes (Rifkind 1964; Diwan et al. 2007; Schweers et al. 2007; Zhang et al. 2009a; Grosso et al. 2017). These mono-ribosomes form aggregates with ferritin and mitochondria to exhibit net-like (reticular) aggregates that stain blue in the presence of supravital stains such as cresyl blue (hence, the name reticulocytes) (Bessis and Breton-Gorius 1962). The removal of ribosomes in erythroid cells involves an *Ulk1*-dependent, *Atg-7*-independent process (Schweers et al. 2007; Mortensen et al. 2010; Moras et al. 2017).

The marginal bands

Marginal bands were first reported in the nucleated cell of chick embryo and termed "Randreifen" (Dehler 1895). The marginal bands are bundles of 5 to 25 microtubules which reside at the circumferential edge of the erythrocyte. Each microtubule measures from 20 to 28 nm in diameter and is stabilized by tau-like proteins and syncolin (Sanchez and Cohen 1994; Murphy and Wallis 1983; Murphy and Wallis 1985). The marginal bands are thought to regulate and maintain the shape of the erythroid cell (Maser and Philpott 1964; Cohen and Terwilliger 1979; Joseph-Silverstein and Cohen 1984; Cohen 1978). Marginal bands have been reported in foetal rats, mice, humans, foetal and adult rabbits and camels (Grasso 1966; van Deurs and Behnke 1973; MIURA et al. 1974; Cohen and Terwilliger 1979). The erythrocytes of camels, which are maintained by marginal bands, resemble erythrocytes from humans with elliptocytosis (Cohen and Terwilliger 1979). More recent studies failed to detect marginal bands in mice and lampreys (Repasky and Eckert 1981; Hagerstrand et al. 2009). This is presumably because of a lytic medium used in all studies performed by Cohen and his colleagues for isolating and visualizing marginal bands (Cohen 1978; Cohen and Terwilliger 1979). This raises the question if marginal bands truly exist in erythroid cells in vivo.

Peroxidase activity and granules

Peroxidase activity in bone marrow erythroid cells was reported in guinea pigs, rabbit and humans (Dvorak et al. 1972; Higashi et al. 1953; Breton-Gorius 1975; Breton-Gorius and Reyes 1976). Peroxidase activity could be detected in the cytoplasm, heterochromatin of erythroid erythroblasts and reticulocytes and nuclear matrix of erythroblasts (Dvorak et al. 1972; Higashi et al. 1953; Breton-Gorius 1975; Breton-Gorius and Reyes 1976). Other cytoplasmic organelles, such as the Golgi apparatus, endoplasmic reticulum, siderosomes, rhopheocytosis vacuoles and mitochondria, lack peroxidase activity (Breton-Gorius 1975; Breton-Gorius and Reyes 1976). Pleomorphic granules containing acid phosphatase in the Golgi area of proerythroblasts exhibit peroxidase activity (Breton-Gorius and Reyes 1976). Azurophilic granules with catalase activity are adjacent to the perinuclear cisternae of maturing erythroblasts (Breton-Gorius 1975). These azurophilic granules are absent in reticulocytes.

Step 4: Surface membrane protein sorting

Surface molecule redistribution during enucleation

During enucleation, the expelled nucleus is surrounded by a thin layer of cytoplasm and cell membrane. Some authors term this structure a "pyrenocyte" coming from the ancient Greek words "pyrenos" for stone of a stone fruit, and "kytos" for empty vessel or cell (McGrath et al. 2008b). Prior to the asymmetric separation of the reticulocyte from the pyrenocyte, a set of cell membrane molecules have been observed preferentially partitioning onto either what will become the reticulocyte membrane and what will become the pyrenocyte (Isern et al. 2008). This protein sorting process is critical in the establishment of a reticulocyte with few remaining adhesion molecules (Lee et al. 2004). Interference with this process appears to occur in a number of haematological disorders including spherocytosis (Salomao et al. 2010).

Cell surface adhesion molecules are partitioned onto the pyrenocyte membrane including α4, α5 and β1-integrins, CD9, CD44 and CD147/Basigin (Isern et al. 2008; Fraser et al. 2007b). In contrast, a number of highly glycosylated, putative anti-adhesion molecules are sorted onto the reticulocyte membrane including the Ter-119 antigen, Glycophorins A and C, Rhesus antigen and the bicarbonate exchanger SLC4A1/Band 3/AE-1 (Lee et al. 2004; Salomao et al. 2010). In an elegant mass spectroscopy-based study, Bell and colleagues analysed peptides derived from FACS-purified human reticulocytes or nuclei (Bell et al. 2013). Similar to observations made examining mouse erythropoiesis, β1-integrin was preferentially sorted onto the pyrenocyte (Bell et al. 2013). In contrast to the mouse, Band 3 and CD44 were found on both cellular components (Bell et al. 2013). SLC4A1 has been observed on bovine erythroblasts, reticulocytes and pyrenocytes (Yeo, Bahrami, Fraser-personal communication). Bell and colleagues also found that the reticulocyte preferentially contains cytoskeletal elements such as spectrin, actin, adducin, myosin and protein 4.1. Endoplasmic reticulum proteins were found enriched in the nuclear fraction (Bell et al. 2013). The patterns of protein sorting observed between reticulocytes and pyrenocytes are summarized in Table 2.

How are surface molecules preferentially sorted onto the reticulocyte or the pyrenocyte membranes? A number of these surface molecules have either direct or indirect connections to the skeleton. Band 3 is well characterized as both a surface bicarbonate exchanger and a component of the erythroid cytoskeleton. During enucleation, cytoskeletal components such as spectrin, actin and adducin are maintained within the reticulocyte, keeping Band 3 and partners upon the reticulocyte surface. The *nb/nb* mutant mice produce a truncated form of the cytoskeletal protein ankyrin and also exhibit reduced sorting of GPA onto the reticulocyte membrane (Lee et al. 2004). Band 3 forms an ankyrin-binding surface molecule complex along with protein 4.2. GPA and Rhesus antigen can also interact with ankyrin in distinct complexes (Satchwell et al. 2015; Nicolas et al. 2006). Protein sorting is also aberrant in the mouse model of protein 4.1-deficient elliptocytosis (Salomao et al. 2010). Gautier et al. (2016) has recently surveyed the distribution of 1318 proteins, including 87 membrane proteins, between human pyrenocytes and reticulocytes by quantitative proteomics. Red cell membrane proteins are predominately observed in the reticulocyte, but a small subset of proteins, such as β1-integrin or CD36, are even specifically sorted to the pyrenocyte. This systematic approach will likely generate new hypotheses on mechanisms underlying protein sorting between these two cell types.

Step 5: Nuclear expulsion

Expulsion of the nuclei from orthochromatophilic erythroblasts is termed enucleation. During enucleation, approximately 40 pg of nuclear material is being removed (Klinken 2002). This equates to nearly 7 g of erythroid DNA expelled daily in a healthy adult human, 2.5 kg in a single year. It has been estimated that erythroid cell enucleation relieves the heart of pumping 1000 t of erythroid nuclei per day (Klinken 2002). The process of nuclear expulsion is a form of asymmetric cell division resulting in a reticulocyte and a pyrenocyte and occurs within the erythroblastic island niche. Erythroid cells must exit the cell cycle to commence condensation and enucleation. Klf1 orchestrates the exit of orthochromatic erythroblasts from the cell cycle. *Klf1*-deficient erythroblasts condense their nuclei but maintain cell cycling and almost uniformly fail to enucleate (Gnanapragasam et al. 2016; Gnanapragasam and Bieker 2017).

Table 2 Redistribution of erythroid proteins during reticulocyte-pyrenocyte maturation. This table very broadly summarizes numerous studies exploring the preferential sorting of proteins to the pyrenocyte or the reticulocyte, as well as studies investigating the internalization, clustering and exocytosis of molecules from the reticulocyte (references given in main body of text)

Molecules sorted onto pyrenocyte	Molecules sorted onto reticulocyte	Molecules released from reticulocytes by exocytosis
Membrane: Integrins, CD44, CD147	Membrane: CD71/Tfr1, Glycophorins, Epb4.2, SLC4A1, Rhesus antigen, CD47, flotillins, aquaporins	Membrane: integrins, CD44, CD71/Tfr1, aquaporins
Nucleus: condensed pyknotic nucleus enzymes related to nuclear function Histones and lamins	Cytoskeleton: Spectrin, ankyrin, adducin, tubulin, vinculin, tropomodulin, talin	Exocytosis: Actin, tubulin, organelle remnant, vesicle-related proteins

Eccentricity of the condensed nucleus

Firstly, the erythroid cell must be made flexible. Vimentin intermediate filaments form a cytoskeletal network that holds the nucleus in place (Koury et al. 1989). During erythropoiesis, vimentin is highly expressed and organized into intermediate filaments. During erythropoiesis, vimentin is downregulated and the intermediate filaments lost (Sangiorgi et al. 1990). This leads to the nucleus becoming eccentric and polarized in mammalian erythroid cells. In comparison, in avian erythrocytes, vimentin is upregulated during erythroid maturation and the intermediate filaments strengthened (Granger et al. 1982; Granger 1982; Sangiorgi et al. 1990). Polarization of the condensed nucleus, mediated by microtubule rearrangement, is essential for nuclear extrusion (Brinkley 1985; Mardin and Schiebel 2012). The condensed nucleus is first polarized eccentrically in process regulated by microtubule-dependent phosphoinositide 3-kinase (PI3K) (Wang et al. 2012; Skutelsky and Danon 1967; Simpson and Kling 1967; Skutelsky and Danon 1972). This polarization is also mediated by the motor protein, dynein, which mediates unidirectional movement of cargo towards the minus end of microtubule (Kobayashi et al. 2016). Trim58, an E3 ubiquitin ligase highly expressed during late erythropoiesis, degrades dynein after the erythroid nucleus is polarized (Kobayashi et al. 2016).

Figure 2 illustrates the process of enucleation.

Enucleation: Getting rid of the cargo

While PI3K signalling and dynein motor proteins are involved in erythroid nucleus polarization, neither plays a role in erythroid nuclear extrusion. Cyclin-Dependent Kinase-9 (CDK-9), which regulates the cell cycle, also regulates nuclear condensation and enucleation, though the mechanisms behind this are unknown (Wölwer et al.). During enucleation, centrioles are found either near the nucleus at regions where expulsion is occurring or at the opposite pole of the cells, distal from the polarized nucleus (Skutelsky and Danon 1967). A cytokinetic-like furrow (or contractile actinomyosin ring) forms in the region between the extruded nucleus and the incipient reticulocyte mediated by F-actin and microtubules regulated by Tropomodulin 1 and Gelsolin (Cantù et al. 2012; Skutelsky and Danon 1972; Skutelsky and Danon 1967; Koury et al. 1989; Simpson and Kling 1967; Nowak

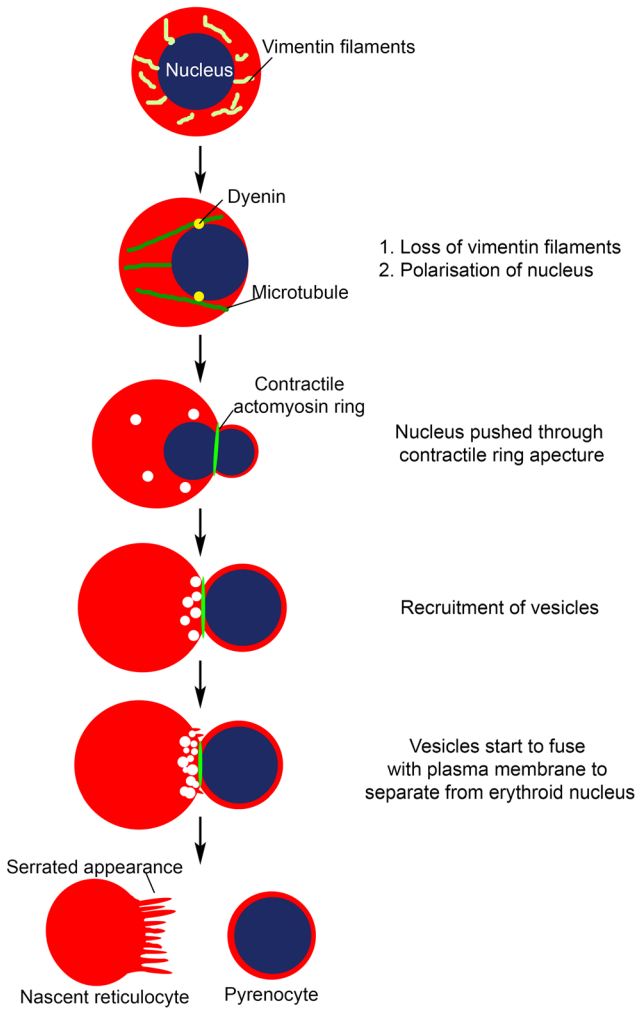

Fig. 2 Schematic description of the process of erythroid enucleation

et al. 2017). F-actin, distal from the nucleus, generates cytoplasmic contractions and hence, pressure to push the elastic nucleus through the narrow aperture out of the cell (Skutelsky and Danon 1967; Nowak et al. 2017). Vesicles or vacuoles are then trafficked between the incipient reticulocyte and nucleus. These vesicles coalesce into bigger vesicles, promoting remodelling of the plasma membrane and separation of the erythroid nucleus (Simpson and Kling 1967; Keerthivasan et al. 2010; Keerthivasan et al. 2011). This result in the production of a serrated reticulocyte and a small, nucleated cell with a thin rim of cytoplasm referred to as the extruded erythroblast nucleus or the pyrenocytes (McGrath et al. 2008b; Keerthivasan et al. 2010). Enucleation can be viewed as a form of asymmetric cell division. However, analysis of mice lacking well-characterized regulators of neuroblast asymmetric cell division led to the conclusion that neither Par3, Scribble nor Pins/Gpsm2 play roles in erythroid enucleation (Wölwer et al. 2017).

Step 6: Nucleus engulfment and destruction

Pyrenophagocytosis

Pyrenocytes display phosphatidylserine (PS) on the surface of the cell membrane that surrounds the condensed nucleus. PS then serves as the major signal for macrophages to engulf the pyrenocyte. Tim4 is a PS receptor expressed on erythroblastic island central macrophages (Miyanishi et al. 2007). Interference with PS-Tim4 binding by MFG-E8 prevents pyrenocyte engulfment by erythroblastic island macrophages (Miyanishi et al. 2007). The same research team that found Tim4 was a receptor for pyrenocyte PS also found that the receptor tyrosine kinase Mer (also known as Mertk) was second receptor for pyrenocyte PS though only in the presence of Protein S, a serum protein (Toda et al. 2014).

Pyrenocytolysis: Pyrenocyte destruction by erythroblastic island macrophages

With approximately 2 million new reticulocytes released per second in a healthy adult human, an appropriately catabolic system must be put in place to manage the vast amount of nucleic acid expelled during enucleation. Skutelsky and Danon performed transmission electron microscopy (TEM) on sections of spleens from lethally irradiated mice which were attempting to recover from the anaemia induced by radiation (Skutelsky and Danon 1967). This study elegantly revealed the pyknotic nature of mature erythroid expelled nuclei and the presence of these nuclei within splenic macrophages found in clonal regions. The central macrophage of EBIs engulfs and destroys these expelled erythroid nuclei. Using a fluorescently labelled transgenic mouse system, Isern and colleagues observed progressively degraded erythroid nuclei within the macrophages of foetal liver macrophages (Isern et al. 2008). Expelled nuclei were observed in foetal liver macrophages and these showed clear signs of degradation following internalization (Isern et al. 2008). A similar transgenic approach revealed uptake of embryonic erythroid nuclei by placental macrophages (Vacaru et al. 2013).

Deletion of the gene encoding DNAse II leads to embryonic lethality in utero (Kawane et al. 2001). This is a consequence of rupture of foetal liver macrophages which were rendered incapable of degrading the large numbers of engulfed erythroid nuclei. DNAse II is an endonuclease important in the degradation of nuclei. As well as playing a role in the foetal liver macrophages, DNAse II also helps in enucleation of another cell type, the lens epithelial cells of the eye, which must be devoid of all cellular contents to allow light through the lens (Yoshida et al. 2005). The transcription factor Klf1/Eklf, which is primarily expressed by erythroblasts, is also expressed in EBI-CM. Indeed Klf1 binds to the *DNase2a* promoter and activates transcription in EBI-CM (Porcu et al. 2011).

The pyrenocyte consists of a thin phospholipid bilayer, a small amount of cytoplasm including haemoglobin and the condensed erythroid nucleus (McGrath et al. 2008b). The heme present in the pyrenocyte haemoglobin is thought to be degraded by heme oxygenase 1, which is strongly expressed by erythroblastic island central macrophages (EBI-CM) (Fraser et al. 2015). Heme oxygenase contains heme into CO2, free iron and biliverdin. Heme oxygenase-1-deficient mice lack functional erythroblastic islands. The few EBI that have been isolated from *Hmox1*-deficient bone marrow showed a highly abnormal domed morphology with few erythroid cells attached (Fraser et al. 2011; Fraser et al. 2015). Figure 3 shows several pyrenocytes engulfed by an EBI-CM.

Step 6: Reticulocyte maturation into erythrocytes

Following enucleation, a distinct cell type arises, the reticulocyte. Reticulocytes are characterized as lacking nuclei, possessing a small number of remnant mitochondria and containing polyribosomes and RNA species. Reticulocytes can be observed interacting with EBI-CM in islands isolated from the bone marrow (An and Mohandas 2011; Yeo et al. 2016)(Fig. 3). The points of contact between EBI-CM and reticulocytes are narrower than those between erythroblasts and EBI-CM. Here, we will touch on the processes related to reticulocyte maturation with an emphasis on the EBI microenvironment.

Ribosomes and RNA species

Reticulocytes possess considerable amounts of mRNA. Thiazole orange, a fluorescent RNA-binding probe, can be used to distinguish reticulocytes from erythrocytes by flow

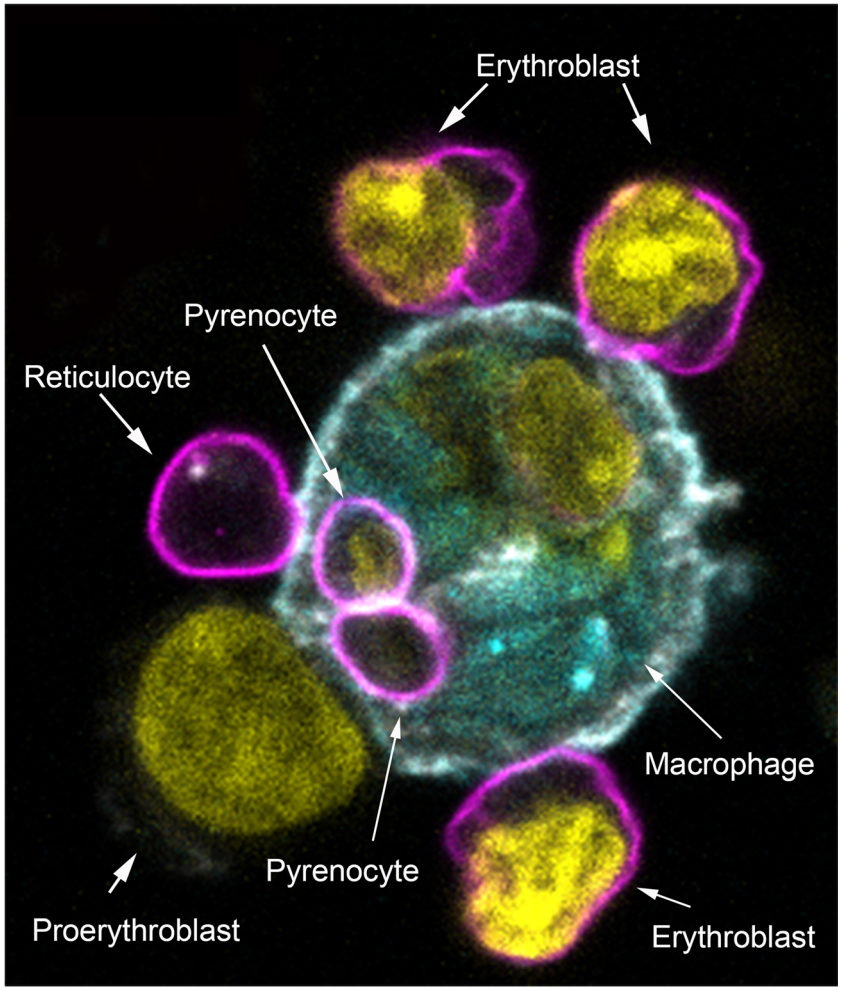

Fig. 3 Single confocal plane image shows pyrenocyte engulfment and reticulocyte attachment to an erythroblastic island central macrophage. This image is a single plane of focus from the maximal projection image of a domed macrophage shown in Fig. 1. Ter119 (magenta) is a surface protein surrounding erythroblasts (with yellow nuclei), a reticulocyte and two pyrenocytes within the macrophage (cyan). Phalloidin in grey illustrates F-actin redistribution during pyrenocyte engulfment

cytometry (Corberand 1996; McGrath et al. 2008a). The vast majority of the mRNA species present in reticulocytes encode globin proteins to increase the intracellular amount of haemoglobin present in the reticulocyte as it matures into an erythrocyte. The mRNA species present in reticulocytes are translated by ribosomes which are still present within the reticulocyte. A small number of mitochondria can also be observed within reticulocytes. These perdurant mitochondria are removed from the reticulocyte by the ongoing process of mitophagy. In mice lacking the mitophagy-related gene *Nix*, reticulocytes are released into circulation bearing greater numbers of mitochondria compared to wild-type mice (Schweers et al. 2007). This in turn leads to an anaemic state in the *Nix*-deficient mice (Schweers et al. 2007).

Surface membrane dynamics

As mentioned above, reticulocytes can still be found interacting with EBI-CM in EBI (Yeo et al. 2016)(Fig. 3). Hence, enucleation and the concomitant loss of adhesion molecules through protein sorting do not lead to immediate detachment from the EBI. A small fraction of original adhesion molecule expression perdures on reticulocytes. This includes $\alpha 4\beta 1$, CD44 and other adhesion molecules (Patel et al. 1985) (Blanc and Vidal 2010). CD71/Tfr1 is also present on the reticulocyte surface at high levels. These surface molecules must be removed from the surface during maturation of reticulocyte into the mature erythrocyte (Patel et al. 1985; Liu et al. 2010b). CD71 is removed from the cell surface by clathrin clustering of surface proteins (including perdurant adhesion molecule expression) followed by endocytosis (Liu et al. 2010a). These endosomes then fuse into larger exosomes which are then released. Using a proteomics approach, Moura and colleagues identified non-muscle myosin II as an important regulator of autophagosome function in reticulocytes (Moura et al. 2018). Exosome-driven loss of surface molecule expression also takes place in non-enucleating species such as the chick, suggesting that this process is distinct from the machinery driving enucleation. Within 48 h in the circulation, reticulocytes lose their spheroid shape and become biconcave discoids via cytoskeletal remodelling. Reticulocyte-derived exosomes (affectionately termed Rex) have been isolated from human cord blood reticulocytes. Proteomics analysis of Rex showed presentation of a range

of surface molecules including CD71/Tfr1, integrins, solute carriers as well as proteins linked to the organelles recently targeted for destruction (Díaz-Varela et al. 2018). Mass cytometric analysis of 31 different surface markers during reticulocyte-erythrocyte maturation confirmed the loss of perdurant adhesion molecules and CD71/Tfr1 (Chu et al. 2017; Díaz-Varela et al. 2018). The expression of CD71 correlated to Mitotracker signal suggesting the CD71+ reticulocytes maintain some mitochondrial activity but by the time CD71 is lost from the surface of the reticulocyte, mitochondria are lost (Díaz-Varela et al. 2018).

Changes in reticulocyte volume

The endocytic process described above results in the loss of adhesion molecules and CD71 and a simultaneous loss of ~ 20% of the reticulocyte membrane as well as cytosolic tubulin and actin (Blanc and Vidal 2010; Ovchynnikova et al. 2018a). The water channel aquaporin-1, which regulates cell volume, is also removed from the reticulocyte surface by endocytosis (Blanc et al. 2009). Other transporters play a role in the reduction of reticulocyte cell volume including the potassium-chloride co-transporter KCC (Quarmyne et al. 2011). Activation of KCC during reticulocyte-erythrocyte maturation leads to the loss of water from the reticulocyte (Quarmyne et al. 2011). The mechanosensory Piezo1 channel has also been implicated in the regulation of erythrocyte volume (Gallagher 2013). Mutations in the gene encoding Piezo1 have been shown to result in the red blood cell disorder hereditary xerocytosis (Zarychanski et al. 2012). Xerocytosis, meaning "dry cell condition" is due to the gradual leakage of K^+ and Na^+ ions from the cell leading to loss of water from the cell and dehydrated erythrocytes.

Cytoskeletal dynamics during reticulocyte to erythrocyte maturation

Having expelled their nuclei, reduced attachment from EBIC M by adhesion molecule sorting and then migrated into the circulation, the final step for erythrocyte formation requires the remodelling of the reticulocyte cytoskeleton (Liu et al. 2010a, b; Ovchynnikova et al. 2018). The reticulocyte membrane is unstable compared to the mature erythrocyte cell membrane (Waugh et al. 2001). A number of cytoskeletal components are preferentially sorted into the reticulocyte including spectrin, the adducins, ankyrin and SLC4A1/Band 3, the transmembrane bicarbonate exchanger. Spectrin forms the main portion of the reticulocyte and erythrocyte cytoskeleton with ankyrin forming junctional complexes with transmembrane proteins such as SLC4A1, Glycophorin A, Rhesus antigen and CD47. Tubulin and actin are removed through exocytosis or ubiquitination and proteosome degradation (Beck and Nelson 1996; Liu et al. 2010a, b).

What is the evolutionary advantage of erythroid enucleation?

All mammalian species enucleate their erythroid cell: monotremes, marsupials and eutherian mammal erythrocytes all circulate without a functional nucleus. In contrast, the erythrocytes of birds, reptiles, amphibians and fish all possess nuclei. Recent analysis of fossilized theropod bones suggested that dinosaur erythrocytes are larger and ovoid—similar to avian erythrocytes and hence are potentially nucleated (Bertazzo et al. 2015). What then could be the evolutionary pressure that led to proto-mammals investing considerable energy and cell machinery in developing a system to expel the nucleus from all erythroid cells? One suggestion is that the more deformable mammalian erythrocytes could move through smaller vessels. Smaller, more deformable erythrocytes could move through smaller blood vessels, extending the range of oxygen transport more deeply into tissues. There is a correlation between erythrocyte size and vascular size; however, it has recently been shown that non-mammalian cynodonts had an almost identical relationship between erythrocyte and microvascular size compared to modern mammals (Huttenlocker and Farmer 2017). The rheology of erythrocytes varies between nucleated and enucleated species. Duck erythrocytes become more viscous in microvessels than mammal erythrocytes—a less favourable situation for oxygen transport and flow rate (Gaehtgens et al. 1981).

One major advantage of not having a nucleus is the lack of susceptibility to viral infection. Reptiles and birds are all vulnerable to infection with viral infections leading to erythroblastosis and erythroleukaemia. In contrast, erythroleukemia in humans is a very rare presentation. A lack of nucleus and the transcriptional and translational machinery present in a nucleated cells results in erythrocytes lacking in expression of MHC class I or II proteins and hence not antigen presentation. This could leave enucleated erythrocytes vulnerable to infection by parasites or bacteria, as is the case in malaria. However, avian erythrocytes can be infected by malarial plasmodium in spite of possessing nuclei. The loss of nuclei in mammals has led to the suggestion that the heart needs to pump less as the mass of the same number of erythrocytes is less in enucleated species. This is likely to be a fortunate by-product of the initial evolutionary pressure to enucleate—which currently remains unknown.

Outstanding questions

We have aimed to give an overview of the biophysical processes at play in the red blood cell-producing niche, the erythroblastic island. We have also aimed to highlight the gaps in our understanding of the process of erythropoiesis. Ranging

from aspects of regulation of the volume of the nucleus, through to how proteins are preferentially sorted, and to how macrophages cope with the massive amounts of DNA they engulf daily, our understanding of erythropoiesis has only just scratched the surface.

For example, epigenetic regulation is likely to be essential for the regulation of the gene expression regime throughout erythroid differentiation as well for pyknotic condensation of the chromatin prior enucleation. Despite decades of intensive research, these two processes have not been clearly segregated. In addition to the lack of temporal information on histone modification, we do not have a detailed overview on the post-translational modification of histones, apart from H3 and H4. Are epigenetic modifications of the chromatin alone sufficient for nuclear condensation and extrusion? There is a fast influx of calcium into the orthochromatic erythroblasts, and the calcium accumulation persists throughout the enucleation process (Wölwer et al. 2016). As calcium ions induce chromatin compaction, it would be interesting to examine the role of calcium, and other cations, in erythroid nuclear condensation (Phengchat et al. 2016). Furthermore, a systematic investigation into the roles of protein phosphorylation in erythroid enucleation is urgently needed.

We have little understanding of the nature of the macrophage at the centre of erythroblastic islands. Is this a unique type of macrophage? What are the ontogenic origins of these macrophages and are they physiologically unique compared to other macrophage lineages? What is the role of EBI-CM in iron metabolism? Does EBI-CM recycle the cellular components obtained by the destruction of millions of pyrenocytes? Proteomics analyses of different stages of erythropoiesis have shed light on the processes of protein sorting, enucleation and reticulocyte maturation. However, the evolutionary advantage of engaging in these energy-intensive processes is not clear.

Concluding remarks

Over 2 billion people are thought to suffer from some form of anaemia. Developing a better understanding of how erythrocytes are formed is more important than ever and has the potential of improving the lives of a significant percentage of the human population. Whether the pathology is due to as-yet undefined mutations, dietary deficiency, infectious disease or chemical toxicity, understanding how humans can produce a vast number of functional, mature red blood cells is of critical importance. The niche in which this massive cellular production takes place was discovered in the 1950s and is ripe for exploration with the remarkable technological advances in single molecule imaging, electron microscopy, single-cell omics and genetic modification.

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Compliance with ethical standards

This work was performed with appropriate ethical approval.

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Conflict of interest Jia Hao Yeo declares that he has no conflict of interest. Yun Wah Lam declares that he has no conflict of interest. Stuart T. Fraser declares that he has no conflict of interest.

Ethical approval This work was performed with the appropriate animal ethics committee (University of Sydney) approval.

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