Erythroblast enucleation

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Erythrocytes, commonly called red cells, are the cellular elements of blood that perform the unique function of ensuring proper oxygen delivery to the tissues.[1] The average blood volume for an adult is 5 liters (55-75 mL/Kg of body weight) and the blood contains approximately 10^9 red cells per milliliter. Red cells do not normally contain a nucleus and are unable to proliferate. They have a limited life-span (~120 days in humans) and are replenished by the constant generation of new cells from hematopoietic stem/progenitor cell compartments. The process of erythropoiesis includes two phases: a first commitment/proliferation phase in which stem/progenitor cells are induced by extrinsic (growth factors) and intrinsic (transcription factors) factors to expand and to activate the differentiation programs and a second maturation phase in which the first morphologically recognizable erythroid cell (the pro-erythroblast) becomes unable to proliferate and undergoes cytoplasmic and nuclear alterations.[1] Cytoplasmic maturation includes loss of mitochondria, reduction of ribosome numbers and reorganization of the microfilament structure and is mediated by the autophagic program, a proteosome-dependent pathway of proteolysis developed by eukaryotic cells to survive starvation (but which may lead to death).[2] Nuclear changes involve chromosome condensation and loss of cytoplasmic-nuclear junctions in preparation for enucleation and may represent an extreme case of asymmetric division (Figure 1).

The enucleation process

The earliest recognizable erythroid cell, the pro-erythroblast, undergoes four or five mitotic divisions which generate, in sequence, basophilic, polychromatophilic and orthochromatic erythroblasts (Figure 2A). The morphological differences between these cells reflect progressive accumulation of hemoglobin (and other erythroid-specific proteins) and decrease in nuclear size and activity.[1] The nucleus becomes dense, because of chromosome condensation, is isolated from the cytoplasm by a ring of cytoplasmic membranes and moves to one side of the cell.[3] The orthochromatic erythroblast is then partitioned into two daughter structures, the reticulocyte, containing most of the cytoplasm, and the pyrenocyte, containing the condensed nucleus encased in a thin cytoplasmic layer. This partitioning is called nuclear extrusion or enucleation and is favored by interaction between the erythroblasts and the macrophage within the erythroid niche, an anatomical structure first identified by Bessis in 1958[4] (Figure 1). Since most of the pyrenocytes are engulfed and degraded by the macrophage,[3] their recognition as *bona fide* cells occurred when they were discovered in the blood of embryos (which contains limited numbers of macrophages) where they are released during the enucleation process of primitive mammalian erythroblasts.[5]

Enucleated erythrocytes are present in the blood of all mammals, suggesting that enucleation provides an evolutionary advantage. Studies in lower eukaryotes (budding yeast and Drosophila) are clarifying that the nucleus is encased within the cytoplasm by microfilaments that bridge the nuclear membrane with the plasma membrane[6] (Figure 1). In addition, although acquiring a relaxed conformation in interphase, the proteins of the mitotic spindle retain their connection with the chromosomal centrosomes.[7] This protein mesh encases the cell into a rigid scaffolding framework which reduces cell deformability but ensures that during mitosis both nuclear and cytoplasmic contents are appropriately partitioned in the two daughter cells. The profound changes in structural membrane protein synthesis (such as band 3, band 4.1 and α- and β-spectrin) and loss of microfilament protein synthesis (vimentin) occurring during erythroid maturation destroy plasma-nucleus

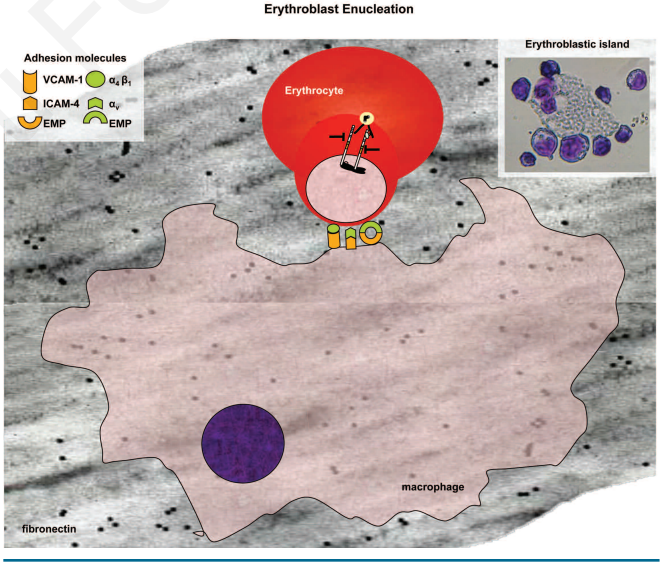
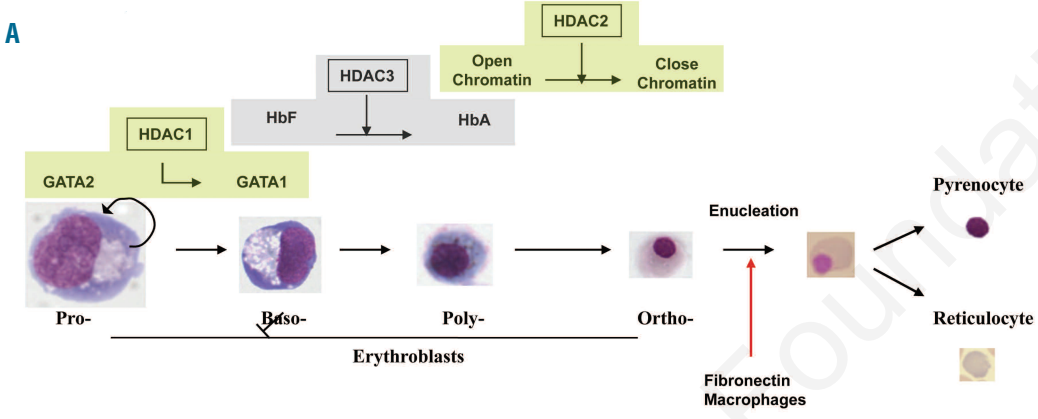


Figure 1. Diagrammatic scheme of the interaction between an erythrocyte and a macrophage during the process of enucleation. The alterations in the structural proteins of the plasma membrane and of the cytoplasmic filaments occurring during erythroid maturation disrupt the connection between the nucleus, the plasma membrane and the centrosome. The centrosome, therefore, becomes unable to drive dissolution of the nuclear membrane, to organize the fiber-chromosome spindle or to apply the tension necessary to divide the cell into two distinct elements. It is speculated that the tension necessary to divide the erythrocyte into a reticulocyte and a pyrenocyte is applied by the macrophage (or the fibronectin mesh) through cell-cell interactions with the pole of the erythrocyte membrane containing the receptors required for macrophage interaction.[3] The insert shows an erythroid island formed by human erythroid cells and a macrophage at day 10 of *ex-vivo* culture (magnification 40X). The background is an electroimmuno-gold staining with fibronectin-specific antibody (the dots) of marrow mesh from a mouse (magnification 20,000X). The adhesion molecules mediating the interactions between erythroid cells and the macrophage are drawn from Chasis *et al*. The diagram of the cytoskeleton elements connecting the centrosome (yellow circle) with the nuclear membrane and with the chromosome centromere are from Razafsky *et al*. These connections are interrupted in erythrocytes.

connections in preparation for enucleation.[1] These structural changes may be advantageous because, by decreasing cell rigidity, they facilitate the passage of red cells through the microvasculature and may minimize cardiac workload.

Asymmetric divisions in which the genetic and cytoplasmic components are differentially partitioned between daughter cells play a key role in the regulation of differentiation. Since cells divide along a plane orthogonal to the centrosome-spindle axis and the fibers of the spindle are linked to the cytoplasmic membrane, polarization of the cytoplasmic components with respect to the centrosome provides an anatomical basis for asymmetric partitioning of all cell components. In the case of the erythrocyte, loss of physical interaction between the nucleus and the cytoplasm may allow an extreme asymmetric division in which all the cytoplasm is inherited by one cell (the reticulocyte) and all the nuclear content by the other (the pyrenocyte), providing a mechanism to increase the concentration of



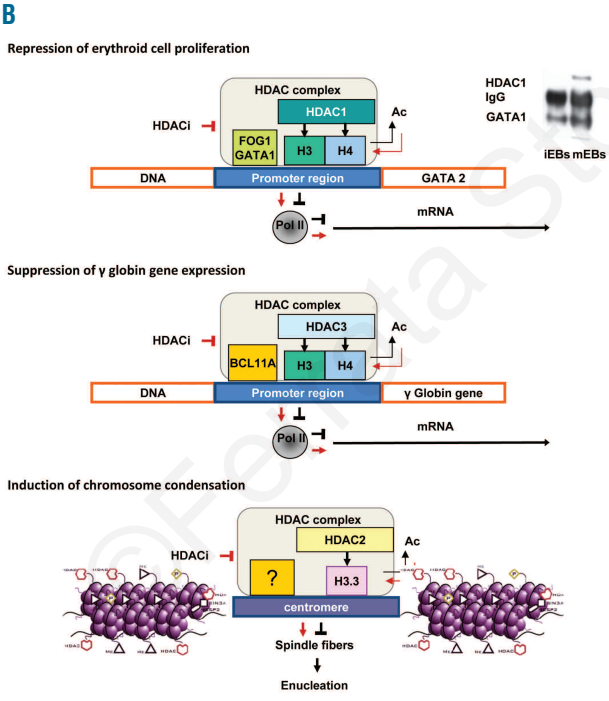


Figure 2. HDAC isoforms (A) and HDAC complexes (B) involved in erythroid maturation. The earliest recognizable erythroid cell, the proerythroblast, is capable of self-replication and of maturation into basophilic erythroblasts. Once nuclear condensation is completed, the orthrochromatic erythroblast undergoes enucleation, a process that generates two daughter structures, the reticulocyte, which contains most of the cytoplasm, and the pyrenocyte, which contains the nucleus surrounded by a small cytoplasmic ring. HDAC1, HDAC3 and HDAC2 have been identified to regulate the decision between self-replication and maturation, the switch from γ- to β-globin expression and chromatin condensation in preparation for enucleation, respectively. (i) Cell proliferation: two GATA transcription factors, GATA2 and GATA1, control proliferation and maturation of erythroid cells.[17] When expression of GATA2 is greater than that of GATA1, erythroid cells proliferate while when GATA1 expression becomes predominant, cells mature. GATA2 and GATA1 regulate each other's expression. GATA2 activates GATA1 expression while GATA1, once expressed, up-regulates its own expression and suppresses that of GATA2. GATA1 suppresses GATA2 expression by docking to the regulatory region of the gene a complex containing HDAC1.[18] By deacetylating the histones, the complex closes the chromatin configuration of the locus which is no longer recognized and transcribed by the polymerase complex. Biochemical studies coupled with loss of function studies in the mice have identified that GATA1 binds the complex indirectly through its obligatory partner FOG1 which contains a binding domain for HDAC1.[18] The insert in (B) shows an immuno-precipitation with a GATA1-specific antibody of protein extracts from *ex-vivo* expanded immature (iEBs) and mature (mEBs) human erythroblasts analyzed by western blot for the presence of GATA1 and HDAC1. These data confirm that GATA1 and HDAC1 are also associated in mature human erythroblasts, suggesting that this complex may suppress GATA2 expression (and proliferation) also in these cells.

(ii) Hemoglobin switching: the observation of specific histone acetylation patterns during globin switching in mice has suggested that HDAC may participate in the silencing complex that represses γ-globin gene expression during erythroid maturation.[v] Proof-of-concept for HDAC involvement in repression of γ-globin expression was further provided by the observation that the HDAC inhibitor (HDACi) butyrate delays the HbF to HbA switch in sheep fetuses and induces HbF synthesis in human erythroid cultures, in adult baboons, in some patients with β-thalassemia and in most patients with sickle cell disease. siRNA-mediated loss of function experiments have recently indicated that the isoform which specifically suppresses HbF synthesis in human erythroid cells and that is targeted by butyrate is HDAC3.[19] More recent genetic and mass spectrometry studies have identified that the specificity of the silencing may be provided by recruitment to the complex of BCL11A which docks the HDAC to the γ-globin regulatory region.[20] (iii) Chromatin condensation: in this issue of the journal Ji *et al.*[10] describe that chromatin condensation in preparation for orthochromatic erythroblast formation is regulated by HDAC2. It is possible that, by deacetylating the centromere-specific histone H3.3,[7] HDAC2 is also responsible for dissociation of the centromeres from the fibers of the spindle. The docking protein for HDAC2 to the centromere has not been identified yet.

hemoglobin (Hb) and other functional proteins in the reticulocyte which may also be evolutionarily advantageous. Disruption of the centrosome motor does, however, make the process of cell division dependent on tension provided by interactions with external elements such as macrophages and/or fibronectin (Figure 1).

Role of histone deacetylases in epigenomic regulation of erythropoiesis

Chromosome condensation is the ultimate form of epigenomic regulation in which all the chromosomes become organized in heterochromatic structures.[7] The shift of chromatin from "open" (euchromatin) to closed (heterochromatin) configurations is determined by the acetylation status of histones H3 and H4.[8] The histone acetylation status is regulated by two enzyme superfamilies, the histone acetyltransferases (HAT) and the histone deacetylases (HDAC) which catalyze, respectively, histone acetylation and deacetylation inducing open and closed chromatin configurations.[9] Eighteen distinct mammalian HDAC, grouped into four classes depending on their primary homology to the *Saccharomyces cerevisiae* deacetylases, have been reported.[9] HDAC function as multiprotein complexes with transcription factors, which ensure specificity by docking the complex to appropriate consensus sequences, and protein kinases, which modulate the activity by altering phosphorylation status. Each HDAC is recruited into a specific complex, suggesting that each isoform may control specific cell functions. The regulation of HDAC isoform expression and assembly in functional complexes in erythroid cells is still poorly understood. Evidence has emerged that HDAC1 regulates proliferation and that HDAC3 regulates switching from fetal (F, containing γ-globin) to adult (A, containing β globin) Hb (Figure 2).

A newly identified role for histone deacetylases in erythropoiesis

The study by Ji *et al.*[10] published in this issue of the Journal provides the first detailed analyses of the expression of different HDAC isoforms during maturation of murine erythrocytes. Previous investigators had already identified that HDAC are required for chromatin condensation prior to enucleation of murine erythroblasts immortalized with the Friend virus.[12] Using primary normal cells, Ji *et al.* confirmed these data by demonstrating that the HDAC inhibitors trichostatin A and valproic acid inhibit chromatin condensation of primary erythroblasts in culture. Using short interfering RNA technology, they then identified that the process of chromatin condensation is specifically dependent on HDAC2 activity while HDAC1, 3 and 5 are apparently dispensable (Figure 2). This study not only increases our knowledge on the role of specific HDAC isoforms in erythropoiesis but also suggests that anemia is a possible side effect of treatment with HDAC inhibitors.

Recent advances in translational research on histone deacetylase inhibitors

The clinical use of HDAC inhibitors ranges from hemoglobin F activators for hemoglobinopathies to inhibitors of cancer growth and infectious diseases.[9,12] The first clinical use of an HDAC inhibitor (suberoylanilide hydroxamic

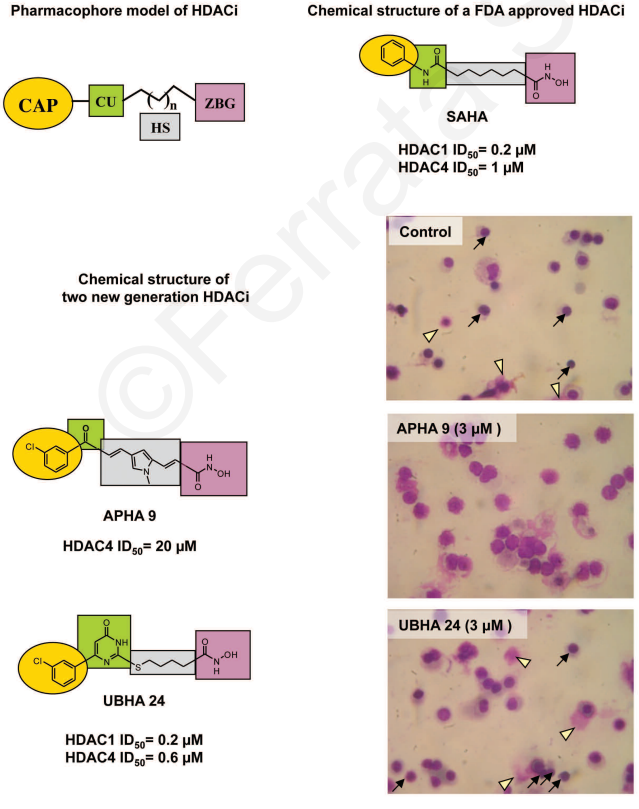


Figure 3. Pharmacophore model of HDAC inhibitors (HDACi) and the chemical structures of a FDA-approved HDACi (SAHA) and of two representative new generation HDACi. The effects of these two compounds on enucleation of human erythroblasts cultured in the presence of erythropoietin are presented on the left. A compound inhibits HDAC activity by irreversibly binding to the catalytic domain of the enzyme. To bind to the catalytic domain, its chemical structure should resemble either the substrate (the acetylated lysine of the proteins) or the product (the acetate ion) of the enzymatic reaction. The pharmacophore model for HDACi, such as SAHA or trichostatin A, which mimic the structure of the substrate includes four domains: a zinc binding group (ZBG), a hydrophobic spacer (HS), a connection unit (CU) and an interaction domain with the rim of the catalytic pocket of the enzyme (CAP).[9,12] By altering the chemical residues of these domains, pharmaceutical chemists are synthesizing new generation HDACi, such as the compound aroyl-pyrrolyl hydroxyl-amide 9 (APHA 9) and uracyl-based hydroxyl-amide 24 (UBHA 24) described in this Figure. The inhibitory activity (ID_{50}) of SAHA, APHA 9 and UBHA 24 against purified human HDAC4 and HDAC1, used as examples of class II and class I HDAC, is reported, for comparison. APHA 9 and UBHA 24 were identified by screening a library of 24 new HDACi for their ability to reactivate γ-globin expression in erythroblasts generated *ex-vivo* from normal donors and from β°-thalassemic patients.[16] The paper by Ji *et al.*[10] led us to perform re-analyses for signs of enucleation in May-Grunwald-Giemsa stained smears prepared from cells obtained in the course of this previous study. Pyrenocytes (arrows) and ghosts of reticulocytes (arrow-head, reticulocytes do not survive the shear force of the centrifugation process) were easily detectable on smears of human erythroblasts induced to mature with erythropoietin for 4 days (control). Nuclear condensation and enucleation were, instead, greatly inhibited by addition of APHA 9, suggesting that HDAC are also required for chromatin condensation of human erythroblasts. However, the presence of UBHA 24 had no apparent effect on enucleation of human erythroblasts in culture. These results indicate that it should be possible to identify therapeutically active HDACi which may not induce anemia because they do not target HDAC2 and do not suppress enucleation. Magnification: 40X.

acid, SAHA, vorinostat, Zolinza®) was approved by the Food and Drug Administration in 2006 for cutaneous T-cell lymphoma. Numerous additional HDAC inhibitors are currently in phase II or III clinical trials (alone or in combination) for the treatment of various tumors. HDAC inhibitors are also in clinical trials for infectious diseases, such as *Candida albicans* (to prevent parasite adherence to host cells), human immunodeficiency virus (to reactivate latent virus and facilitate its eradication by antiviral therapies) and malaria (to inhibit the *Plasmodium falciparum* life cycle).

HDAC inhibitors may also have applications in regenerative medicine. Treatment with trichostatin A or valproic acid in combination with forced expression of Oct4, Sox2, Klf4 and c-myc greatly increases the efficiency with which mouse and human somatic cells are reprogrammed into induced pluripotent cells which may be used to generate autologous cells for therapeutic purposes.[17] The demonstration that *ex-vivo* expanded red cells protect mice from lethal bleeding[14] has suggested that red cells generated *ex-vivo* from induced pluripotent cells may represent alternative products for autologous transfusion in humans.[15] The data from Ji *et al.*[11] however, indicate that erythrocytes expanded from HDAC inhibitor-treated induced pluripotent cells may not be suitable for transfusion because they may fail to enucleate.

Given this wide range of clinical applications, there is an enormous effort to design new, possibly more potent, HDAC inhibitors.[12] The recognition of isoform-specific HDAC functions has provided a paradigm-shift for the design of HDAC inhibitors. The aim of current studies is to increase clinical efficacy by identifying the HDAC isoform to be targeted and then designing HDAC inhibitors specific for that isoform[9,12] This search has been facilitated by the availability of crystallographic data on the binding of the catalytic domain of bacterial HDAC with trichostatin A which led to the development of a pharmacophore model for HDAC inhibitors[9] (Figure 3). Based on this model, new generation HDAC inhibitors have been synthesized and are currently in clinical trials. We have identified two new HDAC inhibitors with different class specificity, both of which activate γ-globin production in erythroid cells expanded *ex-vivo* from normal donors and β-thalassemic patients.[16] As predicted by Ji *et al.*, APHA 9 prevented chromosome condensation and enucleation of human erythroid cells in culture but UBHA 24 did not (Figure 3). This observation predicts that further studies on the biological activity of new generation HDAC inhibitors (possibly involving crystallographic data of the binding domain of HDAC inhibitors with individual human HDAC isoforms) will, in the near future, enable the identification of therapeutically active compounds that, by not affecting HDAC2 activity, should not induce anemia.

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