Concise Review: Advanced Cell Culture Models for Diamond Blackfan Anemia and Other Erythroid Disorders

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ABSTRACT

In vitro surrogate models of human erythropoiesis made many contributions to our understanding of the extrinsic and intrinsic regulation of this process in vivo and how they are altered in erythroid disorders. In the past, variability among the levels of hemoglobin F produced by adult erythroblasts generated in vitro by different laboratories identified stage of maturation, fetal bovine serum, and accessory cells as "confounding factors," that is, parameters intrinsically wired in the experimental approach that bias the results observed. The discovery of these factors facilitated the identification of drugs that accelerate terminal maturation or activate specific signaling pathways for the treatment of hemoglobinopathies. It also inspired studies to understand how erythropoiesis is regulated by macrophages present in the erythroid islands. Recent cell culture advances have greatly increased the number of human erythroid cells that can be generated in vitro and are used as experimental models to study diseases, such as Diamond Blackfan Anemia, which were previously poorly amenable to investigation. However, in addition to the confounding factors already identified, improvement in the culture models has introduced novel confounding factors, such as possible interactions between signaling from cKIT, the receptor for stem cell factor, and from the glucocorticoid receptor, the cell proliferation potential and the clinical state of the patients. This review will illustrate these new confounding factors and discuss their clinical translation potential to improve our understanding of Diamond Blackfan Anemia and other erythroid disorders. STEM CELLS 2017; 00:000–000

SIGNIFICANCE STATEMENT

By contrast with theoretical science, experimental science tests models which by definition are imperfect because they contain "confounding factors." In the past, confounding factors identified in the in vitro models developed to study human erythropoiesis has greatly contributed to advance the field. Recent technological advances have greatly improved the power of analyses provided by in vitro models of human erythropoiesis. However, these improved models contain novel "confounding factors" that have yet to be fully discussed. This review will illustrate three novel confounding factors wired in advanced in vitro models of human erythropoiesis and discuss how their identification may drive additional studies to improve our understanding of the pathobiology of Diamond Blackfan Anemia and possibly other erythroid disorders.

INTRODUCTION

In vivo studies on the regulation of human erythropoiesis and how it is altered in disease states is challenged by ethical considerations that limit the manipulations to which human beings may be subjected. In the 1970s, this recognition led to the development of surrogate assays for human hematopoiesis represented by semisolid cultures in which human bone marrow cells were induced to generate colonies composed of mature blood cells [1]. These assays, as imperfect as they were at that time, were instrumental to move the field forward. As examples, they provided the biological basis that guided the identification, biochemical purification, and genetic cloning of human hematopoietic growth factors [2] and, coupled with genetic investigations, allowed demonstration of the clonal nature of some malignant hematopoietic disorders, such as polycythemia vera [3]. Establishing these in vitro models represented such a great success in itself that criticism on how accurately they predict in vivo situations was not fully explored. The fact that cells and proteins identified by these models exerted in vivo

the expected biological functions was considered sufficient to validate their use.

First Realization That In Vitro Models Are Sensitive to "Confounding Factors"

In the 1980s, the availability of erythropoietin (EPO) purified from the sera of polycythemic sheep, the by now forgotten step-III EPO [4], made possible the use of in vitro assays for identifying the abnormalities of hemoglobin production in patients with hemoglobinopathies [5]. These studies soon recognized that erythroid progenitor cells from nondiseased adult individuals have the ability at the single cell level to generate in vitro erythroid cells that produce fetal (F) hemoglobin [6]. However, the ranges of hemoglobin F detected in these cells by different laboratories varied widely (from 0% to 1% up to 15%) [7–9]. To reconcile this variability, a systematic analysis of the culture components was conducted to identify which of them could affect hemoglobin F production. These parameters, or as currently defined "confounding factors" [10], were represented by "stage of cell maturation," EPO concentration and presence of macrophages and of compounds contained in selected batches of fetal bovine serum [11–14]. In particular, fetal bovine serum was recognized as a source of factors, some of which were removable by charcoal treatment, which could exert either negative or positive effects on hemoglobin F production [15–17].

The recognition that in vitro models contain intrinsic biases did not discourage some investigators who were instead inspired by them to develop novel areas of research. As examples, the confounding factor "stage of maturation" lead to design therapies for hemoglobinopathies based on drugs that accelerate terminal maturation, such as hydroxyurea [18]; the identification of the regulatory effects of macrophages fostered the development of in vitro models to study the erythroid bone marrow niche (Supporting Information Movie S1) [19–21] while the factors present in fetal bovine serum, by providing proof-of-principle that in adult cells hemoglobin switching is sensitive to microenvironmental cues, inspired studies aimed toward the identification of signal transduction elements that could be targeted to increase hemoglobin F production in patients with hemoglobinopathies [22, 23].

Other investigators instead considered in vitro modeling as lacking rigor and developed holistic approaches represented by mouse models to study human hemoglobin switching that were instrumental to the identification of the regulatory regions of the human β-globin locus [24, 25].

Improvement of In Vitro Models for Human Erythropoiesis

In the 1990s, the cloning of stem cell factor (SCF, also known as Steel factor or KIT ligand) [26–28], which in combination with EPO regulates red blood cell production in vivo [29], greatly facilitated our ability to model human erythropoiesis in vitro. In liquid cultures supplemented with optimal concentrations of SCF and EPO, and suboptimal concentrations of interleukin-3 (IL-3), human hematopoietic progenitor cells undergo synchronous unilineage differentiation which generates erythroblasts by days 10–12 and red blood cells by days 17–20 [30, 31]. These cultures are considered models for steady-state hematopoiesis because growth factors are used at concentrations that, although higher than those present in human plasma, may reflect those present in the bone marrow. In the bone marrow, SCF is expressed by the niche in a membrane-bound form which is 1–2-logs more potent than the soluble form used in culture [32], IL-3 is produced locally by the adipocytes [33], and EPO establishes synergies with components of the stroma which improve its bioavailability [34]. Steady-state modeling generates 5,000–50,000 erythroid cells per progenitor cell, numbers which are sufficient for most but not all biochemical studies.

Studies in mice on the recovery from hemolytic anemia induced by phenyl-hydrazine, which has represented for years the gold standard to measure EPO concentration in biological fluids [35], led to the recognition that recovery from stress requires in addition to EPO [36] increased levels of glucocorticoids, hormones produced by the adrenal gland and released in blood [37]. Glucocorticoids exert their functions by binding to the glucocorticoid receptor (GR) and activate the stress response in all mammalian cells [37]. The clinical observation that patients with gain (Cushing's syndrome) or loss (Addison's disease) of GR function develop, respectively, erythrocytosis or anemia [38, 39] indicated that GR regulates stress erythropoiesis also in man. This recognition inspired the development of liquid cultures stimulated with SCF, IL-3, and EPO plus a synthetic GR agonist, either hydrocortisone or dexamethasone (Dex), which is 10-fold more active, as surrogate models for human "stress erythropoiesis" [40–42]. In these models, differentiation remains unilineage but the erythroid cells from the BFU-E [43–45] to the proerythroblast stage [46], express a set of GR-induced genes (such as ZFP36L2) which retains them into a self-renewal state blocking progression to terminal maturation [43, 44, 47]. By days 10–14, stress-specific cultures generate erythroblasts that, although similar in morphology and pattern of CD71/CD36/CD49d/CD235a expression to those generated in cultures without glucocorticoids [48, 49], represent distinctive cell populations that should be more properly defined stress-specific erythroid cells. In fact, in addition to expressing GR-induced genes [43, 44, 47], these cells differ from those generated in the absence of Dex for lacking MPL (the receptor for thrombopoietin) and gaining TGF-β receptor 3 expression [46, 50] and for being responsive to BMP-4 [51, 52] and not to thrombopoietin [46]. When transferred to differentiation cultures containing EPO, insulin, transferrin, and thyroid hormone, stress-specific erythroblasts, as those obtained without Dex, mature into reticulocytes within 7 days [53]. Culture models of stress erythropoiesis generate great numbers ($>10^5$ cells per progenitor cell) of erythroid cells starting from limited volumes of blood and are therefore suited for studies involving pediatric patients with congenital erythroid disorders (Fig. 1). As an example, this model has been instrumental to define the sequela of molecular epigenetic events which ultimately leads to the production of red blood cells by sources from different developmental stages (fetal liver, cord blood, and adult blood and marrow) [56, 57].

Rationale Supporting the Use of Improved In Vitro Models to Study Human Erythropoiesis

Restrictions posed by animal welfare activists on mouse experiments have recently revitalized the use of surrogate in
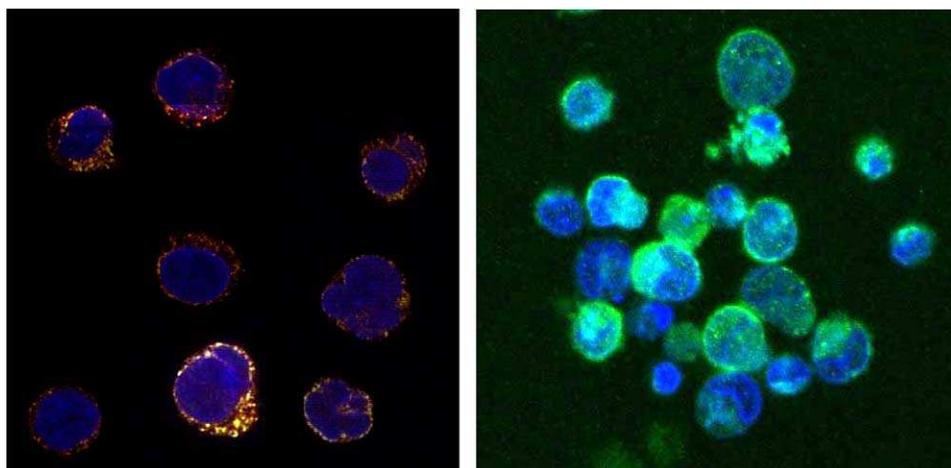
Figure 1.   Examples of clinical applications of in vitro modeling of erythroid cells. Left panel: Representative confocal microscopy image of erythroid cells expanded in vitro from adult blood stained with antibodies against calreticulin (red signal) and the glucocorticoid receptor (GR). These studies may elucidate mechanisms which regulate the stress response in human erythroid cells and how their alterations may determine the pathobiology of myeloproliferative neoplasms [54]. Right panel: Representative confocal microscopy analyses for EKLF (also known as KLF1, green signal) in erythroid cells expanded in vitro from adult blood. Human erythroid cells expanded in vitro may elucidate the crossregulation between EKLF and BCL11A in the regulation of hemoglobin F production as well identify the effects exerted by EKLF mutations in human erythropoiesis [55]. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole. Original magnification ×1500. Scale bars are not presented.

vitro models to study human erythropoiesis. This use has also been fostered by the increasing numbers of reports describing significant differences in the regulation of murine and human erythropoiesis [58]. One of the first differences to be noted is the ability of granulocyte–macrophage colony stimulating factor (GM-CSF, currently known as CSF2) to stimulate erythroid differentiation in culture [59]. In human cultures, GM-SCF stimulates the growth of a subset of erythroid bursts which does not respond to IL-3, while in murine cultures, it only stimulates the growth of granulomonocytic colonies. This difference suggests that the restriction between erythroid and myeloid differentiation is more stringent in murine than in human hematopoiesis. The biochemical basis for these differences is starting to emerge. In fact, the signaling cascade of the EPO receptor includes Ca^{2+} mobilization and the regulator of human erythroid cell expansion (RHEX) factor in human but not in murine erythroid cells genome [60, 61]. The use of improved culture models of human erythropoiesis has been favored by the development of retroviral technologies, which allow altering gene expression or performing gene editing with low numbers of human stem cells.

It has been recognized that also results obtained with these more advanced in vitro models by different laboratories may be widely different. By using sophisticated bioinformatic approaches, a recent commentary by Ulirsch et al. rediscovered that "stage of maturation" is a confounding factor which may affect the interpretation of gene expression analyses and discusses how this factor may bias the interpretation of results obtained using these advanced in vitro models [10]. This commentary ends with the recommendation that conclusions must be supported by results obtained with a uniformly rigorous definition of stage of maturation. However, as pointed out by a related commentary [62], in the case of some diseases, variegation in maturation profile is to be expected because the underlying mutations may alter the expression of the antigens used for its definition. This is the case of, for example, some of the mutations in *EKLF* which impair

expression of the Lutheran antigen [63]. Still, differences observed between these nonperfectly matched cells speak about the mechanisms which had determined the alterations observed.

In addition to maturation stage, however, advanced in vitro models contain novel confounding factors that are still poorly recognized and discussed which may represent both a challenge and a source of inspiration for additional studies. Below, we compared the culture technology used in the latest studies published on Diamond Blackfan Anemia (DBA) to identify some of these novel confounding factors and to discuss how they may inspire areas of research which may increase our knowledge on this and possibly other erythroid disorders.

NOVEL CONFOUNDING FACTORS AND SOME THE AVENUES OF RESEARCH THEY MAY INSPIRE

In muscle cells, GR activates the expression of genes encoding factors that, by suppressing mTOR, reduce phosphorylation of 4E-BP1 and S6K1, inhibiting the early steps (binding to 40S and initiation) of mRNA translation by the ribosome machinery and reducing protein synthesis [64–68]. It is therefore counterintuitive that treatment with glucocorticoids is effective in patients suffering from DBA, a congenital form of pure red blood cell aplasia induced in many cases by genetic mutations that impair the initial steps of mRNA translation by the ribosomes [69, 70]. For reasons still unknown, the treatment is effective only in 60% of the patients and the identification of strategies to cure DBA represents an important unmet clinical need. To identify novel therapeutic targets, scholars have often used surrogate models of human erythropoiesis as tools to obtain a deeper understanding of the pathobiology of the disease. As examples, the discovery that SCF rescues the ability of hematopoietic progenitor cells from DBA to generate erythroid bursts in culture [71–73] inspired an international trial with recombinant SCF that was prematurely interrupted by the National Institute of Health after the death of one patient (Dr. Ramenghi, personal communication) although

Table 1. Summary of the culture conditions and expression results in four of the DBA studies published so far

	Horos et al. [77]	Ludwig et al. [80]	Yang et al. [39]	O'Brien et al. [81]
Sample type	Peripheral blood	Bone marrow	Bone marrow	CD34$^+$peripheral blood
Sample origin	DBA (*RPS19, RPL11*) ($n = 11$)	DBA (RP) ($n = 3$)	DBA (RP) ($n = 3$)	DBA (RP, $n = 5$;GATAl, $n = 2$; unknown, $n = 4$)
	Controls($n = 5$)	Controls ($n = 6$)	Control ($n = ?$)	Control ($n = 24$)
Patient growth	7/11	3/3	3/3	7/11
Culture	One step	One step	Three steps	Two steps (7 + 7 days)
Step 1: Growth factors Glucocorticoids	EPO (0.5 U/ml), murine-SCF (l00 ng/ml) + Dex (l0^{-5} M)	SCF (10 ng/ml), IL-3 (1 ng/ml), EPO (3 U/ml) + Dex (2 × 10^{-5} M)	SCF (100 ng/ml), IL-3 (5 ng/ml), EPO (2 U/ml) + HC (10^{-6} M)	SCF (100 ng/ml), IL-3 (10 ng/ml), EPO (0.5 U/ml), IL-6 (l0 ng/ml), TPO (50 ng/ml) None
Step 2: Growth factors	NA	NA	SCF (100 ng/ml), EPO (2 U/ml)	EPO (3 U/ml)
Step3: Growth factors	NA	NA	EPO (2 U/ml)	NA
Cells analyzed	Day 16	Primary marrow cells	Multiple, up to 17 days	Day 14
Purification	None	CD34$^+$CD71$^+$ CD45$^+$	CD71$^+$CD36$^-$CD235a$^-$ CD71$^+$CD36$^+$CD235a$^+$ CD36$^+$CD235a$^+$CD36$^+$CD235a$^+$	CD44$^+$CD235a$^-$ CD44$^+$CD235a$^+$
Platform	RT-qPCR	Affimetrix U133A	RT-qPCR	Affimetrix human Gene 2.0 ST
ALAS2 mRNA	Not done	No change	Decreased	Decreased
GATA1 mRNA	Not done	No change	Not measured	No change
β-Globin mRNA	Not done	No change	Decreased	Increased
GATA1 targets	Not done	Decreased	N/A	Increased (RP) No change (GATA1)
Heme biosynthesis	Not done	Decreased	N/A	Increased (RP) No change (GATA1)
Ribosome biogenesis	Decreased	Decreased	N/A	Decreased

In addition to the differences reported by Ulirsch et al. [10], some of which are summarized in black fonts, the four studies adopted greatly different improved culture conditions wired with novel confounding factors discussed in this commentary (in red fonts). Modified from [10].

results obtained with some of the patients are available as case reports (see, e.g., [74]). More recently, the observation that DBA contain stress-specific BFU-E that are more susceptible to TGF-β has suggested that galunisertib, an inhibitor of TGF-β receptor 1 signaling under clinical evaluation for a variety of diseases [75], may be an effective therapy for DBA [50].

In an effort to identify novel therapies for DBA, several animal models have been generated. These models are represented by zebrafish, drosophila, and mouse strains genetically modified to carry mutations in ribosomal genes similar to those identified in patients [76]. These models have been instrumental to detail the defective ribosome biogenesis induced by DBA mutations [76]. However, since their phenotype includes only a modest macrocytic anemia which does not require transfusion, they are not recommended for the identification of novel strategies to cure the disease. The search for novel treatment strategies for this disease is therefore still an active ongoing process that heavily relays on the use of in vitro models of the disease and that may be facilitated by critical analysis of the confounding factors present in these models.

INTERACTION BETWEEN SIGNALING FROM cKIT, THE RECEPTOR FOR SCF, AND FROM GR

The first insights on the molecular abnormalities of DBA using ex vivo expanded cells were published by Horos et al. [77]. Using cultures stimulated with murine SCF, EPO, and Dex (Table 1), this study confirmed that CD34$^+$ cells from some but not all DBA patients generate in culture good numbers of

erythroid cells [72] and identified that the mutations in genes encoding ribosomal proteins (*RP*) associated with DBA impair mRNA binding to 40S, providing a mechanism which may reduce mRNA translation in these cells. The recognition that GR activation inhibits mRNA binding to ribosomes in muscle cells [64–68] is a clear indication that the presence of Dex in these cultures represents a confounding factor. However, the cultures contained murine SCF which is structurally similar (83% identical by BLAST search) [78] to the human growth factor but unable to activate the SCF-dependent ERK signaling that protects GR from lysosomal degradation in human erythroid cells [79]. Therefore, human erythroid cells grown with murine SCF are unlikely to contain levels of GR sufficiently high to elicit a Dex response. Whether synergy between cKIT and GR signaling underlies the improvements in erythroid differentiation observed in cultures of DBA stimulated with SCF [71–73] remains an open question.

The observation that RP-deficiency prevents mRNA binding to ribosomes was confirmed in 2014 by Ludwig et al. [80] (Table 1). This group also reported that impairment is restricted to a subset of erythroid mRNA which include that encoding the long form of the transcription factor GATA1 which is specifically transcribed at late stages of maturation [80]. By identifying a DBA patient carrying a loss-of-function *GATA1* mutation and by performing *RP* loss-of-function and *GATA1* gain-of-function experiments with primary mobilized CD34+ cells, this study provided compelling evidence that reduced translation of GATA1 mRNA is responsible for differentiation abortion and insufficient red cell production in DBA. However, the cultures used in this study were stimulated with

Dex in combination with human SCF, which assures that GR is optimally expressed. Therefore, these results are biased by the possibility that inhibition of mRNA binding to the ribosomes was, at least in part, the consequence of GR activation [64–68]. This question was finally clarified in a non-confounding fashion by O'Brien et al. who, using cultures not supplemented with Dex, confirmed that erythroid cells derived from DBA patients carrying *RP* mutations express an abnormal ribosomal signature [81] (Table 1). Furthermore, this article identified that similar ribosomal abnormalities are detected in cells generated from patients carrying the *GATA1* mutation establishing a feed-back loop between *GATA1* and the control of its mRNA translation. This provocative conclusion is independently supported by the observation that the hypomorphic *Gata1* mutation induces abnormal RSP14 signature and deficient ribosome biogenesis in mice [82].

The fact that these three studies reached the same conclusion regardless of the presence of Dex makes questionable whether GR exerts the same effects on ribosome biogenesis in erythroid and muscle cells. Since GR agonists improve red blood cell production at least in some DBA patients [69, 70], it is possible that, by contrast with muscle cells, in erythroid cells GR agonists elicit a non-canonical GR signal which improves ribosome biogenesis. Surprisingly, in view of their obvious significance for ribosomopathies, the effects of these drugs on mRNA translation in erythroid cells have not been investigated as yet. Because of their clear clinical relevance, these effects and how they are affected by the regulation exerted by SCF on GR stability deserve to be carefully investigated. These studies may be relevant not only for DBA but also for other ribosomopathies. In fact, human GR is encoded by *NR3C1* located on the long arms of chromosome 5 (5q31) close to the breakpoint of the chromosomal del(5q) abnormality deleted in patients with myelodisplastic syndrome (MDS), a disease also associated with anemia due to mutations in *RP* genes and reduced ribosome functions. Patients with del(5q) MDS often present with EPO-resistant anemia. A retrospective fluorescent in situ hybridization analyses for *NR3C1* in 14 of these patients identified that in 78% of the cases, the breakpoint involved *GR* suggesting that GR haploinsufficiency exacerbates the ribosomal dysfunctions observed in these patients [83].

Patient Treatment State

O'Brien et al. confirmed that CD34$^+$ cells from only a subgroup of DBA patients generate good numbers of erythroid cells in culture and made the original important observation that CD34+ cells from glucocorticoid-responsive patients generate greater numbers of erythroid cells when collected after treatment [81]. This observation suggests that the responsiveand treatment-state of the patients are confounding factors when assessing the growth of DBA patients in culture. In addition, the fact that the cultures did not contain Dex allows speculating that the "greater expansions" observed with in vivo treated cells reflect the frequency in these populations of stress-specific erythroid progenitor cells [50] which may have been increased by glucocorticoids by promoting both commitment from earlier compartments, thanks to their chromatin remodeling effects [84], and self-renewal [43]. This observation is a paradigm shift in our understanding of the pathology and cure of DBA because it moves the

abnormalities induced by the causative mutations and the therapeutic effects exerted by glucocorticoids up to the stem cell level, possibly at the level of the bipotent erythroidmegakaryocytic stem cell recently identify by Velten et al [85]. Since erythroid–megakaryocytic stem cells are extremely sensitive to thrombopoietin, this hypothesis deserves to be further investigated because it suggests that DBA patients may be responsive to the advanced MPL agonists, such as Eltrombopag, which have been proved effective in MDS and in aplastic anemia [86, 87].

Cell Proliferation State

Ludwig et al. reported that primary stem/progenitor cells (CD34^{pos}) from DBA patients express normal levels of 5'-aminolevulinate synthase 2 (ALAS2, the first protein of the heme biosynthetic pathway) mRNA and decreased levels of mRNA encoding other proteins involved in heme biogenesis [80] (Table 1). By contrast, O'Brien et al. reported that the levels of ALAS2 and of other mRNA involved in heme biosynthesis are, respectively, decreased and increased in early erythroid cells (CD44$^+$CD235a$^-$) generated in vitro from DBA patients [81]. The difference between the results reported by the two groups may relate to the different populations analyzed (stem/progenitor cells vs early erythroid cells) and, as discussed by Ulirsch et al. [10], to the poor definition of stage maturation by O'Brien et al. In fact, O'Brian et al. purified their cells on the basis of CD44 expression, a good marker for the maturation of murine erythroid cells [88] but not for that of the human ones [49]. However, it is also possible that the differences are related to the proliferation state of the cells analyzed. In fact, in human erythroid cells, as in normal and leukemic stem cells [89], CD44 expression predicts proliferation potential [46]. Since post-natal CD34^{pos} cells are mostly quiescent while CD44+ erythroid cells are all in cycle [46], it is possible that differences in mRNA levels observed among cell populations isolated with the CD44 marker highlight abnormalities affecting the ability of the cells to proliferate. This hypothesis is consistent with results published by the Abkowitz laboratory that, by defining erythroid maturation using the canonical CD71, CD36, and CD235a markers [90], confirmed that the most immature, and therefore with greater proliferation potential, erythroid cells expanded from DBA are those that express reduced levels of ALAS2 mRNA [91] (Table 1). However, consistently with the greater levels of mRNA encoding proteins of the heme byosythetic pathway reported by O'Brien et al. [81], this study also reported that these cells contain increased levels of ALAS2 protein and of heme and that increased levels of heme are responsible for accelerated cell death [91]. The identification of cell proliferation potential as confounding factor is related to the fact that, by contrast with murine erythropoiesis in which maturation progresses regularly along four terminal divisions, in human cells terminal maturation and loss of proliferation potential are partially uncoupled. In fact, murine CFU-Ederived colonies contain up to 32–64 cells (i.e., 5–6 divisions) while the corresponding human colonies contain as many as 100–200 cells (>7 divisions). The plasticity in the speed with which human erythroid cells lose proliferation potential with maturation may be related to species-specific differences in the speed of recovery from anemia and is also a clinically

relevant area of research which deserves to be further investigated.

CONCLUSION

In addition to "maturation state" and other parameters already identified, ex vivo modeling of human erythropoiesis is prone to new confounding factors which may challenge the comparison of data obtained by different laboratories but, as already demonstrated in the hemoglobin switching field, may also unveil novel clinically relevant biological phenomena. In the case of DBA, and possibly of other diseases associated with ribosomopathy, systematic analyses of these confounding factors may provide hints on the mechanisms which lead to disease manifestation, glucocorticoids response, and identify novel therapeutic targets.

AUTHOR CONTRIBUTIONS

A.R.M. and L.V.: wrote the manuscript, read the manuscript, and reviewed the literature.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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