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Orchestration of late events in erythropoiesis by KLF1/EKLF

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Abstract

Purpose of review—Transcriptional regulators provide the molecular and biochemical basis for the cell specific properties and characteristics that follow from their central role in establishing tissue restricted expression. Precise and sequential control of terminal cell divisions, nuclear condensation, and enucleation are defining characteristics within erythropoietic differentiation. This review is focused on KLF1, a central global regulator of this process.

Recent findings—Studies in the past year have brought a number of proteins that are targets of KLF1 regulation into focus with respect to their roles in terminal erythroid differentiation. Many of these are involved in fine control of the cell cycle at both early (E2F2, Cyclin A2) and later (p18, p27, p19) stages of differentiation, or are directly involved in enucleation (p18, p27). Dramatic biophysical changes controlled at the nuclear lamin by caspase 3 enable histone release and nuclear condensation, while dematin association with structural proteins alters the timing of enucleation. Conditional ablation of mDia2 has established its role in late stage cell cycle and enucleation.

Summary—Transcription factors such as KLF1, along with epigenetic modifiers, play crucial roles in establishing the proper onset and progression of terminal differentiation events. Studies from the past year show a remarkable multifaceted convergence on cell cycle control, and establish that the orthochromatic erythroblast stage is a critical nodal point for many of the effects on enucleation. These studies are relevant to understanding the underlying causes of anemia and hematologic disease where defective enucleation predicts a poor clinical outcome.

Keywords

KLF1/EKLF; erythropoiesis; enucleation; nuclear condensation; cell cycle

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Conflicts of Interest

None.

INTRODUCTION

Establishment of the proper levels of erythrocytes not only is dependent on a controlled expansion of the erythron during hematopoiesis, but also on the ability to efficiently enter the terminal differentiation steps that lead to a fully formed reticulocyte after condensation and extrusion of its nucleus [1,2]. This unique property depends on the proper interplay and levels of trans-acting factors that coordinate these events [3,4]. Among these tissue-restricted integrators are transcription factors that impart global expression patterns whose correct onset, during both early and late stages of erythropoiesis, are critical for generation of the 2 million enucleated red blood cells per second produced in the human adult. Unsurprisingly, the appearance of nucleated red blood cells in circulation is indicative of onco-hematologic disease and poor prognosis [5]. Here, we will be using KLF1 function in direct and indirect regulation of late stage effectors as a jumping off point to review recent studies from the past year related to very late events in erythropoiesis, particularly those that lead to proper chromatin condensation and extrusion of the nucleus.

KLF1 (EKLF; erythroid Krüppel-like factor)

We have learned much about how the hematopoietic lineages are first established, and how networks of general and tissue specific transcription factors coordinate the phenotypic expression patterns specific to each lineage [6,7]. However, the functional integrations important for the final, terminal differentiation steps of erythropoiesis are not as well delineated. In the case of erythroid cells, these result in dramatic morphological changes that ultimately yield the mature, circulating erythrocyte [1,2].

Among these critical global erythroid regulators is KLF1, an erythroid-enriched protein that binds to its DNA consensus sequence (5'CCMCRCCCN) via three C2H2 zinc fingers at its carboxyl end [8]. Biochemical and genetic studies have established its importance for mammalian erythropoiesis and its interaction partners that support its ability to integrate transcription and epigenetic events at target loci [9–11], particularly with respect to the control of switching mechanisms at the β -like globin locus [12,13]. Importantly, mutations in KLF1 lead to changes in globin regulatory patterns that can be advantageous or more sinisterly can lead to chronic anemia [14,15].

Most recently, KLF1 function has been linked to critically important events during terminal erythroid differentiation, most specifically in its regulation of cell cycle targets at early and late times [16**]. Given the global role of KLF1 in erythropoiesis, it is not surprising that almost all of the genes discussed in the review are also adversely affected by disruption in KLF1 (Table 1). This is buttressed by the phenotypes of the mouse and human KLF1-null erythroid cell [17,18], which do not enucleate (Figure 1), and of human congenital dyserythropoietic anemia (CDA) type IV [19], patients of which show a remarkable level of nucleated erythroid cells in circulation [20,21]. CDA type IV has been linked to a monoallelic mutation in the second zinc finger of KLF1.

CELL CYCLE

It has long been noted that erythroid terminal differentiation is marked by a discrete number of divisions as cell replications are reduced to give rise to the enucleated reticulocyte. Although early stages of erythropoiesis are coupled to proliferation of progenitors [22–24], terminal differentiation from erythroid precursors to hemoglobinized erythrocytes requires 3 to 4 cell divisions, during which its morphology and gene expression patterns become quite specialized [25]. At the orthochromatophilic erythroblast stage, its nucleus condenses, moves to one side of the cell, and is lost [26–29]. This continuum is a process that remains incompletely understood.

The importance of cell cycle regulators for this process is well established. For example, one of the defining features of the switch from the proliferative to the differentiating state is the exit from the cell cycle [30–33]. However, details of their sequential control in the process has remained paradoxical, particularly as genes that both accentuate S phase progression (e.g., E2F2, E2F4, cyclins) and enable exit from the cell cycle (e.g., p18, p27, p21) are required. A key observation that suggested the entry to terminal differentiation exhibits a discrete program came from analysis of primary erythroid populations subdivided based on CD71/Ter119 expression (yielding S0–S5 subsets) [34]. It was noted that the number of cells in S phase and the speed of replication are dramatically increased specifically in the transition from S0 (CD71^{lo}/Ter119[–]) to the S1 (CD71⁺/Ter119[–]) set of differentiating erythroid cells, coinciding with their entry into erythropoietin dependence. Importantly, a dramatic drop in p57 CDK inhibitor level was found to be critical for this transition. The percentage of cells in S phase decreases (from 90% in S1 to <20%) soon afterwards (by S4–S5; CD71^{lo}/Ter119⁺). Even though these data were focused on a relatively early stage of differentiation, it was already clear that the replicative properties of these cells, and the levels of cell cycle control proteins, is not homogeneous but is tightly and sequentially regulated.

Cyclin A2 is known to play a critical role in hematopoietic stem cell proliferation [35]. Its more precise function in erythropoiesis was recently illuminated by conditional ablation studies [36*]. Deletion of the gene in the erythroid compartment led to a greater RDW, increased Howell-Jolly bodies, and deficiency in enucleation in spite of normal proliferation and differentiation. Perhaps surprisingly, coupling this with deletion of Cdk2 had no effect, and coupling to loss of p27 led to an incomplete recovery of only some red cell parameters. Ablation at an earlier stage in culture revealed its requirement for BFU-E formation, suggesting a complex dual requirement for cyclin A2 at both early proliferation and late differentiation stages of erythropoiesis.

A recent study [37*] has brought the role of E2F2 into focus, by showing that E2F2 is required to maintain normal blood cell counts (irrespective of cyclinE-Cdk2 activity), and that S phase transit of erythroid cells is slower under stress conditions in its absence. Lack of E2F2 leads to inefficient nuclear condensation and impaired enucleation. Post-translational protein modifications may be important, as a transcriptome analysis of genes targeted by E2F2 during differentiation led to the identification of Citron Rho-interacting kinase (CRIK)

as a potential modulator of cytokinesis during erythroid differentiation, thus providing a nice link between condensation and enucleation.

The role of CDK inhibitors during erythropoiesis can be less straightforward than might initially appear. This notion follows from a recent study [38**] where p19 was shown to be required for terminal erythroid differentiation, yet whose absence didn't alter the cell cycle. Its mode of action, and thus its downstream effects, follow from its regulation of GATA1 protein (not RNA) levels. It accomplishes this by phospho ERK-directed modulation of Hsp70, a protein whose levels and subcellular localization regulate caspase 3-directed proteolysis of GATA1 [39]. Loss of p19 results in increased apoptosis and altered nuclear morphology, but can be rescued by re-expression of GATA1. Interestingly, caspase 3 is also implicated in nuclear structure alterations (see below), and ERK has been shown to be part of an erythroid protein complex with HDAC, GATA1, and KLF1 [40], tying together a number of the proteins discussed in this review.

KLF1 is known to directly regulate many of the aforementioned cell cycle genes (Table 1), again raising the issue of how the seemingly contradictory effects are regulated, as KLF1 is present in renewable erythroid progenitors and their terminal differentiated progeny [41], cell types that exhibit virtually opposite cell cycle properties. KLF1-null erythroid cells were known to exhibit an abnormal cell cycle profile that is stalled at G0/G1 [42,43]. However, this did not fully explain its control at late stages, a question that has been difficult to address in KLF1-null cells, as complications follow from the inability to take advantage of the (KLF1-dependent) erythroid cell surface markers typically used to distinguish differentiation stages.

However, this problem was successfully surmounted by a recent study [16**], where Image Stream flow cytometric technology [44,45] enabled the morphology and size of the cell and the nucleus to be used as a primary monitor of differentiation status. This data suggests a KLF1-dependent biphasic model that subdivides terminal differentiation into two steps: an early, pro-proliferative phase that enables entry into the multiple S phase cycles via positive regulation of E2F2; and a later, anti-proliferative phase that shuts down cycling by positive regulation of (primarily) p18 and p27 cdk inhibitors.

However, a key surprise in these analyses was that simple re-expression of select cdk inhibitors was sufficient to rescue enucleation in the KLF1-null erythroid cell [16**]. Even though hemoglobinization (and presumably other global KLF1 targets) is not recovered, this epistasis result for the first time directly links cell cycle progression to enucleation. In addition, it unexpectedly shows that any of the cell cycle inhibitor proteins are the necessary and sufficient KLF1 targets required for this penultimate stage of morphological differentiation.

The data suggest that KLF1 directs E2F2 expression during the early phase when renewal is critical, particularly when trying to attain the amazingly high level of replicating cells, but then switches to directing expression of CDK inhibitors at the late phase when cell cycle exit is required. A number of KLF1's protein partners are known, and these include corepressors as well as coactivators [11]. Many of these interactions are altered depending on the

modification state of KLF1 (reviewed in [11]). It is easy to envision that the post-translational status of KLF1 is altered during differentiation (perhaps by HDACs [46], see below), and that this has a functional effect on its activity as differentiation proceeds; in the present case, changing its target loci from those that are pro-S phase (e.g., E2F2) to those that enable cell cycle exit (e.g., CDK inhibitors). However, reagents that distinguish modified KLF1 are not yet available to directly address this issue.

CHEMICAL SCREENING

The ability to use cell size and surface marker expression to efficiently enrich specific subsets of differentiating erythroid cells [25] led to a chemical screening of orthochromatic erythroblasts to identify pathways important for the process [47*]. Reassuringly, inhibitors of processes already known to play a role (such as HDAC and proteasome inhibitors) were rediscovered, but in addition, cyclin-dependent kinases were unexpectedly identified as important for enucleation, particularly CDK9. CDK9 has typically been implicated in RNA pol II transcriptional elongation control, including erythroid transcription [48], so it will be of interest to see how this function interfaces with enucleation, perhaps by means of novel mechanistic properties of Cdk9.

NUCLEAR CONDENSATION AND CHROMATIN MODIFICATION

A critical component for successful enucleation is condensation of the nucleus [49]. The importance of histones, and of the proteins that modify them (especially HDAC2 [50]), in this process is perhaps not surprising [51,52]. What had been unexpected was that at least part of the decrease in nuclear size results from extrusion of histones from the shrinking nucleus [53]. The importance of exportin proteins, particularly Xpo7, in this process partly explains this observation [53].

However, the requirement for caspases in at terminal stages remained enigmatic [54]. Recent studies have shed light on their requirement [55**], demonstrating the unexpected and surprising result that caspase 3 is critical for formation of nuclear openings that enable efficient extrusion of histones. These nuclear openings result from discontinuities in lamin in the nuclear membrane, and occur early in erythropoiesis, recurring multiple times during the final differentiation steps. The openings are cell cycle dependent, and in each case enable extrusion of selected histones from the nucleus into the cytoplasm, where they are degraded in a caspase-dependent fashion. These remarkable dynamic morphological changes then cease just prior to the nuclear polarization and enucleation maturation stages. It will be of interest to address how HDAC activity is overlaid on the caspase activity, and how these are functionally distinguished from apoptotic events.

Given the importance of epigenetic control of gene expression, it is not surprising that chromatin modifiers have recently been implicated in terminal erythropoiesis. In particular, Setd8, a mono-methyltransferase that modifies histone H4K20 [56], plays an important role by inhibiting erythroid maturation [57*], resulting in incomplete nuclear condensation and lower enucleation levels [58*]. Its expression is higher in erythroid precursors than other cell types, and knockdown studies show that part of its function involves repressing Gata2 and

thus promoting erythroid maturation [57*,58*]. Its effects on nuclear condensation and extrusion may follow from its established cell cycle-dependent variation in levels [59–61], linking condensation to the limited number of cycles prior to cell cycle exit.

mDIA2

It has long been known that Rho GTPases play a critical role in powering the enucleation process, particularly in formation/nucleation of the F-actin ring that is formed at the boundary between the cytoplasm and the soon to be extruded nucleus [62]. A subset of formin-related proteins bind to Rac proteins, and one in particular, mDia2, functions as an effector protein for enabling actin polymerization, asymmetric division, and efficient enucleation [63].

mDia2 ablation is embryonic lethal [64], so a recent study addressed its importance for erythropoiesis by its conditional knockout via genetic crosses with Mx-Cre or Vav-Cre mice [65*]. These mice exhibited significant anemia, yielding morphologically aberrant red cells of increased diameter (RDW). Importantly, the erythroid precursors were blocked at a late stage, with many binucleate cells seen in the bone marrow at the orthochromatic stage of differentiation. These analyses also revealed a cell cycle block in G2/M phase consistent with reduced proliferation seen in cultures.

DEMATIN (structural)

Cytoskeletal structural proteins necessarily are important for the enucleation process, as the localized actin-myosin ring and its structural partners provide a biophysical substrata upon which to perform vacuole accumulation, nuclear polarization, and eventual extrusion [66–68]. For example, the role of tropomodulin 3, a protein that interacts with tropomyosin and caps the ends of actin filaments is critical for this effect [69], as is the degradation of dynein by the Trim58 ubiquitin ligase [70].

In this context, dematin is an actin binding protein that is critical for erythroid membrane structural stability [71]. Recently [72*], full length disruption of dematin has been shown to lead to severe anemia and altered erythroid membrane stability and morphology due to alteration of spectrin-actin junctions and loss of adducin. But the unexpected result was that enucleation is precociously *enhanced* in its absence. This was observed in definitive erythroid cells both in vivo and after culture. Although to a lesser extent, enucleation was still significantly increased in the heterozygous cells that retain nearly normal membrane properties. This surprising result brings up the concept of active negative regulation of enucleation, a relatively novel concept in the terminal differentiation field, and leads to numerous questions concerning how membrane structure enables and/or inhibits the process.

CONCLUSIONS

Although we have chosen to focus on putative KLF1 targets in this review (Table 1), there are other regulators that have been recently implicated in the control of late stage enucleation events, including transcriptional effectors (e.g., Foxo3a [73]) and chromatin interactors (e.g., Asxl1 [74]). These suggest at a minimum that the details of how

transcriptional and biophysical signals are integrated during the final stages of erythropoiesis are not likely the result of regulation by a single global regulator, but rather accede to multiple inputs that depend on its physical niche (primitive vs definitive, fetal liver vs spleen vs bone marrow). Nonetheless, it is remarkable how many of the morphological changes described above harken back to cell cycle controls that are altered during this process (Figure 2).

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KEY POINTS

- KLF1 directly or indirectly regulates most genes involved in enabling the late stages of erythropoiesis
- Controlled and sequential expression of a number of cell cycle regulators, both S phase inducing and cell cycle inhibiting, are critical for successful enucleation
- Along with those related to cell cycle regulation, new studies have uncovered novel properties involved in nuclear condensation, chromatin modulation, and structural protein regulation of late events

FIGURE 1.

Morphology of murine erythroid cells during expansion and differentiation phases. Cytospins were stained with MGG. Fetal liver erythroid cell cultures were derived from WT (*top*) or EKLF-null (*bottom*) E12.5 embryos. *Left*, cells during the expansion phase are similar, showing an immature blast-like appearance. *Right*, after in vitro differentiation for 3 days, WT cells are maturing and >30% enucleating (pyrenocyte is shown by arrow), while EKLF-null cells do not enucleate. Numerous double-nucleated cells (arrows) are seen in the EKLF-null cultures (Gnanapragasam and Bieker, unpublished; also see ref [16**]).

FIGURE 2.

Overview of recent studies summarized in this review.

TABLE 1

SUBSET OF KLF1 REGULATED GENES

GENE NAME (<i>genetic loci</i>)	EXPRESSION DEPENDENT ON KLF1		EVIDENCE FOR KLF1 BINDING TO GENE
	mouse	human	
Cell cycle:			
<i>E2F2</i>	+	+	+
<i>E2F4</i>	+		+
p18 (<i>CDKN2C</i>)	+	+	+
p27 (<i>CDKN1B</i>)	+		+
p21 (<i>CDKN1A</i>)	+		+
p57 (<i>CDKN1C</i>)			
p19 (<i>CDKN2D</i>)	+		+
Cyclin E (<i>CCNE1</i>)	+		
Cyclin A2 (<i>CCNA2</i>)			
<i>CDK2</i>		+	+
<i>CDK9</i>			
Enzymatic:			
CR1K (<i>CIT</i>)	+		+
<i>HDAC2</i>			
<i>XPO7</i>	+		+
CASPASE 3 (<i>CASP3</i>)	+		+
mDIA2 (<i>DIAP3, DIAPH3</i>)	+		
Structural:			
DEMATEIN (<i>EPB4.9, DMTN</i>)	+		+
<i>TMOD1</i>	+		+
Transcription/chromatin			
<i>FOXO3A</i>	+	+	+
<i>ASXL1</i>			
<i>SETD8 (KMT5A)</i>	+		+

Mouse and human expression data from RNA seq analyses ([18,75]; Gnanaprasagam and Bieker, unpublished). Binding is based on ChIP studies [76–78].