Chapter 6
Expanded Role for EKLF/KLF1 Within the Hematopoietic Lineage

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Abstract Erythroid Krüppel-like factor (EKLF) is a transcriptional activator that was originally identified in 1993 and has since provided a significant window into the carefully orchestrated process of erythroid gene expression, particularly as it plays a critical role in ß-like hemoglobin switching. However, later observations have suggested other roles for this protein. This presentation discusses the lines of evidence that together open up a new hematopoietic horizon for EKLF function.

Introduction

For a number of years my laboratory has been interested in the control of lineage decisions during hematopoiesis, with particular focus on the study of erythroid cell differentiation. We initially took a subtractive cloning approach to this issue, whereby we isolated genes selectively expressed in the murine erythroleukemia (MEL) cell line but not in a monocyte-macrophage (J774) cell line. This led us to the identification of a gene product that contained three TFIIIA-like C2H2 zinc fingers at its carboxy terminus and that we named erythroid Krüppel-like factor (EKLF) because of their high homology to the *Drosophila* gap gene (Miller and Bieker 1993).

Our excitement about this gene product was heightened when we found that its expression is highly restricted to adult bone marrow and spleen and when we realized that the homology of its zinc fingers to a specific subset of C2H2 zinc finger proteins enabled us to predict, from structural arguments, a potential DNA-binding target. This directed us to a conserved element already known to be important for ß-globin gene expression, the CAC box or CACCC element, located ~90 bp upstream of its transcriptional start site. Subsequent molecular and genetic studies have verified that EKLF interacts with this site in vivo, and that EKLF

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plays a critical and necessary role in the switch to adult ß-globin expression during erythroid ontogeny (Donze et al. 1995; Feng et al. 1994; Im et al. 2005; Nuez et al. 1995; Perkins et al. 1995). It does so by directing the onset of ß-globin expression and establishing the proper three-dimensional chromatin structure across the ß-like globin domain, a large region that includes the far upstream locus control region (LCR) (Armstrong et al. 1998; Drissen et al. 2004).

EKLF has been renamed KLF1 and is now the founding member of a large (17-member) family of transcription factors that play diverse roles in differentiation and development (Bieker 2001).

Although most of these and subsequent studies have focused on EKLF's ability to activate transcription, we had been surprised by finding that EKLF also interacts with co-repressor proteins (Chen and Bieker 2001, 2004), suggesting that other roles lay hidden and remained to be discovered. Together, the activation and repression studies strongly suggested that posttranslational modifications of EKLF play an important role in establishing specific protein–protein interactions, and that these could lead to altered functional effects at both the molecular and cellular levels. For these reasons, we continued to probe EKLF function by concurrently undertaking a series of nominally unrelated studies that, in the end, unexpectedly converged and unraveled a deeper function for EKLF during hematopoiesis.

Four Experimental Avenues That Support an Expanded Role for EKLF/KLF1 During Hematopoiesis

EKLF Promoter Analyses

Examination of the onset and expression pattern of EKLF during development revealed that its mRNA first appears at the neural plate stage (~E7.5), where it is strictly localized to the earliest morphologically identified erythroid cells in the blood islands of the yolk sac, followed by a switch to the fetal lever by E10.5 (Southwood et al. 1996). This led to a series of analyses using varied approaches to determine the control elements responsible for this highly restricted pattern of expression. Initially (Chen et al. 1998), transfection of cloned DNA was used to determine that a 950 base pair (bp) region, located just upstream of the transcription initiation site, was sufficient to generate erythroid-specific expression in transient assays (Fig. 1). This region harbors erythroid-restricted DNAse hypersensitive sites, one of which behaves as a strong enhancer. This core region, in conjunction with the proximal promoter—with its important GATA and CCAAT sequences (Crossley et al. 1994)—accounted for EKLF's tissue-specific expression. The importance of this short region was verified in vivo by the use of transgenic mice, where the 950 piece was sufficient to drive reporter (lacZ) expression specifically to the yolk sac and fetal liver (Xue et al. 2004). Importantly, visualization of thin sections from the yolk sac showed reporter expression to be strictly localized to the hematopoietic,

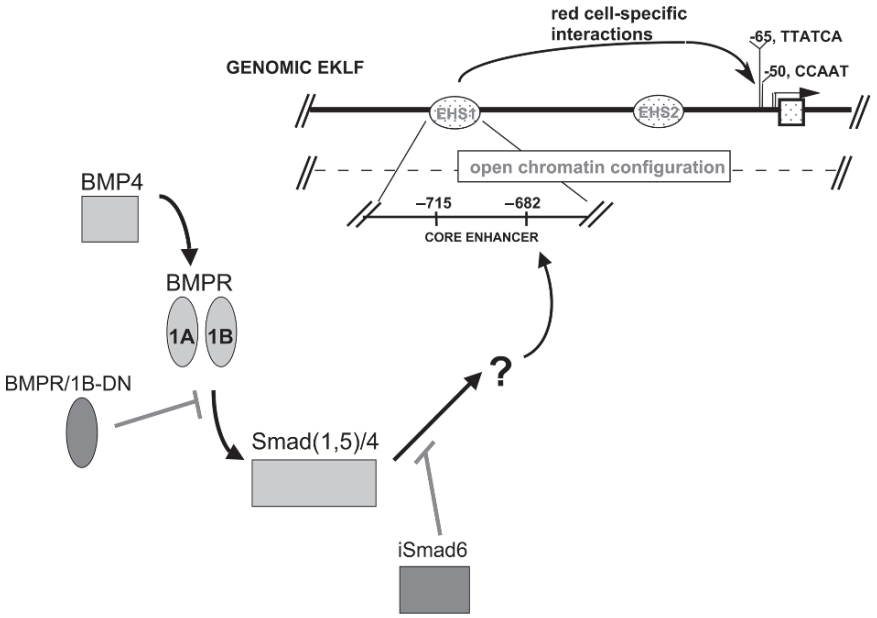

Fig. 1 Regulation of the EKLF promoter, circa 2003. *Top right* The 950-bp region just upstream of the EKLF transcription start site, showing the chromatin structure and location of erythroid hypersensitive sites (*EHS*), conserved promoter motifs, regions responsible for reconstitution of tissue specific expression in transient assays, and the minimal core enhancer element (Chen et al. 1998; Crossley et al. 1994). *Bottom left* Summary of the need for the BMP4/BMP receptor/Smad pathway for EKLF induction, indicating the effects of dominant negative receptor or inhibitory Smad6 on the process (Adelman et al. 2002). The *question mark* in the middle suggests that these experiments had not determined whether the BMP4 pathway exerted its effects on the EKLF promoterin a direct or indirect manner

not the endothelial or visceral endoderm, compartment. Finally, embryonic stem (ES) cells differentiating in serum-free medium were used to show that BMP4 is necessary and sufficient to induce EKLF expression as embryoid bodies (EBs) are being formed (Adelman et al. 2002). The involvement for the Smad pathway in this process was directly implicated by showing that interference by constitutive expression of a dominant negative BMP receptor or of the inhibitory Smad6 obviated EKLF expression even in the presence of serum. Together, these studies suggested that EKLF promoter regulation is controlled by a proximal 950 bp region that responds to BMP4 signals mediated by (likely) Smad1 or Smad5 (Fig. 1). However, the kinetics of its induction did not allow us to determine if the Smad1/5 effect was direct or was mediated indirectly via another protein (or a combination of the two).

This set the stage for a more detailed analysis of the EKLF promoter (Lohmann and Bieker 2008). A seven species alignment of a 30 kB genomic region encompassing the mammalian KLF1 gene indicated that the most significant homology, in addition to the protein coding exons, resided in the same 1 kB proximal promoter

region that had been functionally mapped. A short peak of homology also resided within the first intron. However, a more detailed view of these regions not only revealed transcription factor-binding motifs but also that their architectural layout was exquisitely conserved across species. Of particular interest were Gata and Smad binding motifs, located precisely within the upstream enhancer and proximal promoter sequences previously mapped by functional assays, and within the intronic region. Their functional importance were tested using a novel application of the Kyba/Daley ES/EB differentiating system (Fig. 2A), whereby an EKLF promoter/GFP transgene was developed that faithfully recapitulated the onset of endogenous EKLF expressing during EB differentiation. Mutation of conserved Gata and Smad sites verified that this assay was sensitive enough to distinguish their importance for directing the onset, maintenance, or optimal levels of transcription.

The proteins that interact with these regions were identified in two ways. First, chromatin immunoprecipitation (ChIP) of GATA proteins revealed a switch in their occupancy when comparing early versus late times of EB differentiation, corresponding to GATA2 occupancy in the progenitor stage, followed by GATA1 occupancy after lineage commitment. Second, the Kyba/Daley system was again modified and used to generate a doxycycline-inducible shRNA line (Fig. 2B) directed against Smad5 expression, which verified its critical importance for EKLF expression. Together with the promoter analyses, these data enable us to propose a two-tiered mechanism for transcriptional regulation of EKLF, providing low levels

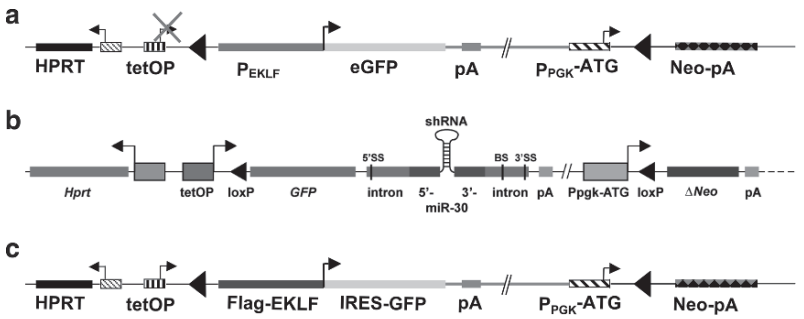

Fig. 2 Modifications of the Kyba/Daley system. The three modifications of the original inducible expression system described by Kyba and Daley (Kyba et al. 2002) used in the experiments described in the presentation are shown. All schematics are shown after their unidirectional, single-copy integration into the single endogenous modified HPRT locus. **A** The EKLF promoter (950 bp; P-EKLF) was placed upstream of the GFP reporter (Lohmann and Bieker 2008). These experiments did not utilize the tet operon but, rather, relied on the EKLF promoter to drive expression of GFP. **B** The miR30 backbone was inserted into an intron in the GFP expression cassette (Lohmann and Bieker 2008). This provides a slot into which any desired shRNA can be inserted; in the present case, they were directed against Smad5. The tet operon is operative and inducible by doxycycline. **C** Flag-tagged EKLF was inserted upstream of an IRES-GFP gene (Frontelo et al. 2007), providing a doxycycline-inducible construct to provide dose-dependent expression of EKLF at any desired time point during embryonic stem (ES) cell differentiation

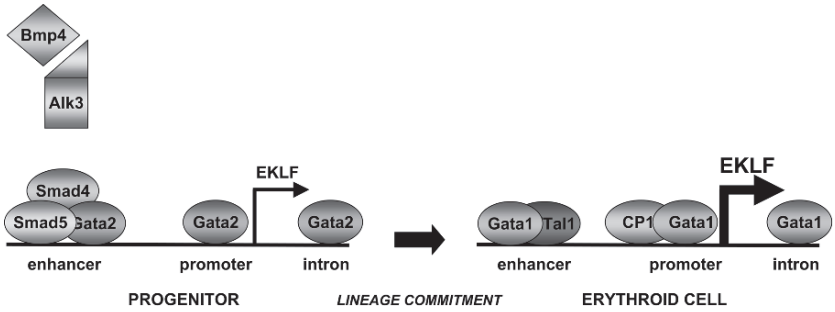

Fig. 3 Model for two-tiered, stage-dependent induction of EKLF during early development. The status of the EKLF promoter is shown before (progenitor) and after (erythroid cell) lineage commitment based on the experiments described in the presentation (Lohmann and Bieker 2008). Multispecies alignments, promoter mutations, ChIP, and shRNA knockdowns suggest that SMAD5 and GATA2 play an initial, necessary role early to generate low levels of EKLF expression, but this switches to a GATA1-dependent, high-level expression pattern after GATA1 protein is induced. Additional proteins (Tal1, CP1, although likely no longer Smad5) may also play a role by binding to other conserved sites

that depend on GATA2 and SMAD5 proteins at early stages, followed by high levels of EKLF transcript after GATA1 protein is produced (Fig. 3).

A prediction from these studies was that selection of EKLF promoter/GFP+ cells should enrich for erythroid progenitors. We therefore established an EKLF promoter/GFP reporter mouse, isolated GFP+ fetal liver cells, and monitored their capacity for colony formation in methylcellulose. Indeed, this selected for erythroid progenitors, but the additional surprise was that this population was also enriched for megakaryocyte progenitors. This was also true when similar assays were performed with EB-derived cells. As a result, there is a line of evidence suggesting that EKLF is positioned by its promoter activity to play an earlier role in hematopoiesis than was originally apparent.

EKLF Gain-of Function and Loss-of-Function Analyses

Although genetic ablation of EKLF led to embryonic lethality due to a profound ß-thalassemia, its expression pattern during embryogenesis (described above) in addition to its presence in some multipotential cell lines suggested other, more subtle functions. In earlier studies, we had used the Kyba/Daley system in its original design for expression of chimeric EKLF proteins (Manwani et al. 2007). Given the enigmatic expression pattern of EKLF cell lines, we used this system (Fig. 2C) to ask whether increased expression of EKLF could alter normal patterns of hematopoiesis during EB differentiation (Frontelo et al. 2007). This powerful system provides a dose-dependent way to increase the target protein at the desired

time point and in all the cells within a developing EB. This approach was combined with disaggregation and plating on OP9 stromal cells. By monitoring cell surface marker expression in cells from EBs that had been induced to overexpress EKLF by the addition of doxycycline, we found that megakaryopoiesis (as judged by CD41/CD42d expression) was repressed. This was not a general effect, as c-kit levels were increased and CD71 levels were not affected. At the same time, however, erythropoiesis was accentuated, as Ter119 levels increased during this time, an effect that was visibly apparent by quantitative morphological examination of the cells.

As a complement to these studies, we monitored megakaryocyte status in EKLF-null fetal livers, harvested at E13.5 just prior to lethality. EKLF-null fetal livers contained a higher percentage of megakaryocytes than did the wild type, judged by both CD41/42d and CD41/42b FACS analyses, which also expanded to a greater extent following cell culture.

We then addressed whether these increased numbers of megakaryocytes resulted from an increase in progenitors. Colony assays using total fetal liver cells, lineage-depleted (lin-) cells, or CMP-sorted cells all led to increased numbers of megakaryocyte colonies if they were derived from EKLF-null material. An unanticipated aspect of these assays was that the EKLF-null megakaryocytic colonies were of greater size than those derived from wild-type material. In total, these data provide a second line of evidence that EKLF functions in hematopoiesis prior to erythroid differentiation, as its levels play a critical role in the extent of megakaryopoiesis.

EKLF Expression During Normal Adult Hematopoiesis

Our earlier studies had focused on EKLF expression patterns during normal mammalian embryonic development but had not addressed this issue during normal hematopoietic differentiation that emanates from the hematopoietic stem cell (HSC). We utilized well-established cell surface marker criteria for isolation of specific subpopulations of cells emanating from long-term hematopoietic stem cells and analyzed them for EKLF presence by reverse transcription-polymerase chain reaction (qRT-PCR) (Frontelo et al. 2007). The analyses (Fig. 4) demonstrated that EKLF is expressed at barely detectable levels in hematopoietic stem cells and multipotent progenitors. A clear difference in expression subsequently becomes established, with EKLF absent in common lymphoid progenitors and their B- and T-cell progeny, yet increased in the common myeloid progenitor (CMP). At this point there is another clear demarcation in expression within the CMP progeny, as EKLF levels become higher in the megakaryocyte/erythroid progenitor (MEP) but decline further in the granulocyte/macrophage progenitor (GMP). EKLF expression in the GMP does not develop any further. These data show that there is a gradual restriction in expression of EKLF as hematopoiesis proceeds even though its levels are steadily increasing in more the differentiated cells that express it. Of particular interest, however, the bipotential differentiation of MEPs leads to a dramatic

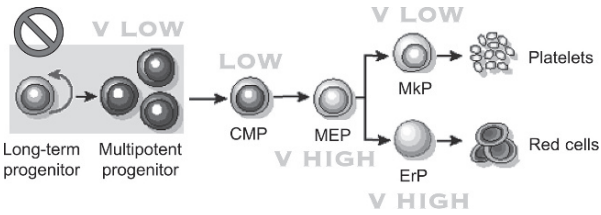

Fig. 4 EKLF expression during normal hematopoiesis. Cell populations from murine bone marrow were sorted and monitored for EKLF expression by a quantitative reverse transcription-polymerase chain reaction, leading to "very low," "low," "high," and "very high" categories based on their relative expression levels. *CMP* = common myeloid progenitor; *MEP* = megakaryocyte erythroid progenitor; *MkP* = megakaryocyte progenitor; *ErP* = erythroid progenitor. Shown are a subset of the total results (Frontelo et al. 2007)

difference in EKLF expression, with erythroid progenitors exhibiting an 80-fold greater level of expression than megakaryocyte progenitors. This demarcates EKLF as having significantly different properties from GATA1, FOG, GFi1b, and SCL—transcription factors whose presence are required for both erythroid and megakaryocytic expansion and differentiation. These data provide a third line of evidence that EKLF is expressed earlier in hematopoiesis and show that it is normally downregulated as MEPs differentiate down the megakaryocytic lineage although being retained at high levels in the erythroid lineage. This also explains the gain-of-function data, which can now be seen as having misregulated the normal shut-off of EKLF in megakaryocytes.

Relevance of EKLF Posttranslational Modifications

EKLF undergoes a range of functionally important posttranslational modifications that encompass phosphorylation (Ouyang et al. 1998), acetylation (Zhang and Bieker 1998), and ubiquitylation (Quadrini and Bieker 2006). Some of these have been shown to alter subsequent protein–protein interactions (Chen and Bieker 2004; Zhang et al. 2001). We noted that mammalian EKLF protein contains a conserved motif near its amino terminus that matches the consensus target site for sumoylation (Siatecka et al. 2007). As a result, we directly tested whether the modification occurs and found that EKLF is sumoylated at a single site within the conserved motif (K74 in the murine sequence), and that PIAS1 plays a critical role in this process. Mutation of this site affects EKLF's repression capability but has no discernible effect on its ability to activate a target promoter. Repression by the wild-type protein can be altered by co-expression of a dominant-negative Ubc9 or the SUMO-specific isopeptidase SSP. EKLF nuclear localization proceeds equivalently irrespective of its sumoylation status. Similar to the other modifications, sumoylated EKLF provides an efficient platform for its protein interactions,

in this case with the Mi2ß subunit of the NuRD repression complex, providing a molecular explanation for its effects in vivo.

When considering the functional importance of EKLF sumoylation, we were fortunate to have obtained, by then, the data on EKLF's ability to repress megakaryopoiesis (summarized above). The data strongly suggested to us that they might provide a means to test whether the two observations are related. We did this in two ways. First, we established K562-derived cell lines that contain stable zinc-inducible EKLF constructs, one with wild type and the other with the sumoylation mutant (K74R). K562 cell lines are an erythroleukemic cell line that can be further directed toward erythropoiesis (fetal hemoglobin expression) by the addition of hemin, or redirected toward megakaryopoiesis by addition of the phorbol ester TPA. Importantly, these cells do not express endogenous EKLF. We found that induction of megakaryopoiesis was inhibited by the presence of wild-type EKLF compared to that seen in the presence of K74R EKLF, which exhibited little inhibition of the process. This suggested that the modification might play an important functional role in megakaryocyte repression.

To further support this idea in vivo during normal hematopoiesis, we cloned EKLF downstream of the megakaryocyte-specific platelet factor 4 (PF4) promoter and established transgenic lines from these DNA constructs. Lines that expressed the transgenes in the bone marrow were examined by cellular and molecular analyses. Already at harvest, bone marrow cells from transgenic mice expressing WT-EKLF displayed a threefold decrease in megakaryocyte cellularity in WT-EKLF transgenic bone marrow. This demonstrates that mis-expression of EKLF in the megakaryocyte lineage has a repressive effect on megakaryocyte formation, consistent with our analyses (see above). However, transgenic bone marrow from the K74R-EKLF line revealed no effect of mutant EKLF transgene expression on megakaryocyte cellularity. We also determined whether transgene expression also altered megakaryocyte colony formation and found a trend similar to that seen with the cell assay: WT-EKLF transgenic bone marrow contained fivefold less megakaryocyte colony-forming potential than the nontransgenic control, and the K74R-EKLF transgenic bone marrow was not affected. We concluded that availability of the SUMO modification site in EKLF is absolutely critical for it to exert its normal inhibition of megakaryopoiesis prior to red blood cell onset and provides the fourth line of evidence for its functional importance earlier in hematopoiesis.

Conclusions and Future Perspectives

These four lines of evidence suggesting that EKLF plays a functional role prior to erythropoiesis leads to a testable working model (Fig. 5). The model includes the results of studies (not presented) supporting the idea that Fli1, an ETS-related transcription factor that is also expressed in the MEP but critical for megakaryocyte differentiation, is negatively regulated by EKLF, providing a molecular basis for EKLF's ability to repress megakaryopoiesis (Frontelo et al. 2007).

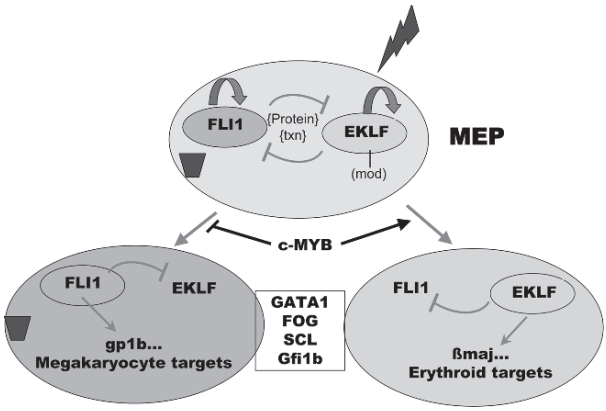

Fig. 5 Model of EKLF's role in erythroid and megakaryocytic decisions. See the text for details.

As a result, we postulate that within the MEP, EKLF and Fli1 can antagonize each other by protein or transcriptional inhibition mechanisms. Stochastic variation in levels, external influences (lightning bolt), or asymmetrically distributed molecules (trapezoid) can enable progeny to attain either an erythroid or a megakaryocyte fate. Also critical is the modification state (mod) of EKLF during these cellular decisions. After cell division, in the erythroid precursor cell EKLF completes the repression of Fli1 while activating downstream red blood cell targets, whereas the converse happens in megakaryocyte precursors as a result of Fli1 suppression of EKLF. Hence, EKLF gain- or loss-of-function leads to divergent effects on the bipotential decision by the MEP. At the same time, and in contrast to the actions of EKLF, other transcription factors (e.g., GATA1, FOG, SCL, Gfi1b) are positively required for both erythroid and megakaryocytic lineages. An interesting exception is c-myb, as hypomorphs have been shown to exhibit effects on MEP bipotential decisions similar to those of EKLF (Mukai et al. 2006).

Recent studies have lent further support for the concepts put forward in this chapter. For example, the role of EKLF in bipotential decisions within the MEP that lead to megakaryocytic repression and erythroid expansion have been supported by directly altering its levels within these progenitor cells and noting their downstream effects (Bouilloux et al. 2008). The importance of single EKLF posttranslational modifications, in this case acetylation of K288, has been shown to be critical for recruiting CBP and the subsequent modification of histone H3, opening the chromatin structure, and transcriptional activation of adult ß-globin (Sengupta et al. 2008). Finally, point mutations in human EKLF have been shown to lead to phenotypic variation of red blood cell antigen expression even in the heterozygous state (Bieker 2008; Singleton et al. 2008). Putting these ideas together leads to the exciting prediction that genetic mutation of EKLF, at sites critical for its protein modification, may in the future be shown to be a causative factor for a specific subset of hematopoietic aberration and disease.

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