Concise Review: Regulation of Self-Renewal in Normal and Malignant Hematopoietic Stem Cells by Krüppel-Like Factor 4

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Key Words. Self-renewal • KLF4 • Hematopoietic stem cells • Leukemia stem cells

ABSTRACT

Pluripotent and tissue-specific stem cells, such as blood-forming stem cells, are maintained through a balance of quiescence, self-renewal, and differentiation. Self-renewal is a specialized cell division that generates daughter cells with the same features as the parental stem cell. Although many factors are involved in the regulation of self-renewal, perhaps the most well-known factors are members of the Krüppel-like factor (KLF) family, especially KLF4, because of the landmark discovery that this protein is required to reprogram somatic cells into induced pluripotent stem cells. Because KLF4 regulates gene expression through transcriptional activation or repression via either DNA binding or protein-to-protein interactions, the outcome of KLF4-mediated regulation largely depends on the cellular context, cell cycle regulation, chromatin structure, and the presence of oncogenic drivers. This study first summarizes the current understanding of the regulation of self-renewal by KLF proteins in embryonic stem cells through a KLF circuitry and then delves into the potential function of KLF4 in normal hematopoietic stem cells and its emerging role in leukemia-initiating cells from pediatric patients with T-cell acute lymphoblastic leukemia via repression of the mitogen-activated protein kinase 7 pathway. Stem Cells Translational Medicine 2019;8:568–574

INTRODUCTION

Stem cells exist at the apex of tissue development and can orchestrate embryonic differentiation of various tissues in an adult organism and regulate tissue homeostasis and regeneration after injury. Embryonic stem cells (ESCs) are the most undifferentiated stem cells and are capable of generating all cell types within the organism, whereas somatic tissue-specific stem cells (e.g., hematopoietic stem cells, HSCs) can only regenerate cells within the same tissue. In bone marrow, HSCs can produce blood cells on demand during homeostatic and regenerative hematopoiesis, and this capacity to produce the blood while maintaining the pool of HSCs is controlled by a delicate balance between self-renewing and differentiating cell divisions. Self-renewal is a specialized and highly regulated cell division producing one or two daughter cells with the same stem cell features as the parental stem cell. This multifaceted mechanism has been the subject of extensive research because of clear implications in tissue homeostasis, regenerative medicine, and cancer therapy. Leukemia is a cancer of the blood cells affecting either lymphoid or myeloid lineages that is caused by genetic and epigenetic alterations occurring in HSC or hematopoietic progenitor cell (HPC); the generated population of leukemic cells bear stem cell properties ensuring self-preservation through their self-renewal capacity while continuously feeding the neoplasm by differentiating into the bulk of leukemia cells. As a critical process regulating stem cell fate in normal and malignant hematopoiesis, self-renewal is controlled by the specialized microenvironment,
or niche, and intrinsic factors that guide the decision of a stem cell to either self-renew or undergo differentiation, depending on the demand of the specific tissue. The Krüppel-like factor (KLF) family of proteins encompasses 17 zinc-finger transcription factors, of which at least 5 have been implicated in key stem cell functions, such as self-renewal [1–5], pluripotency [1–3, 5], embryogenesis [6], and erythropoiesis [7]. Like other members of the family, KLF4 contains activation and repression domains that mediate recruitment of coactivators or corepressors and three fingers that bind to guanine-cytosine-rich sequences such as CACCC found in gene regulatory promoters and enhancers [8, 9]. KLF4 has been increasingly studied since the landmark work describing its role in the reprogramming of somatic cells into induced pluripotent stem cells (iPSCs), and the contribution of KLF4 to this process suggests a potential function in the preservation of stemness in other tissue stem cells [10]. KLF4 is expressed in a wide range of mammalian tissues and regulates diverse cellular processes during normal tissue homeostasis, including proliferation, survival, and differentiation. In fact, KLF4 regulates self-renewal in stem cells from different tissues (e.g., embryonal, intestine, and skin) in both homeostasis and cancer [3, 11–23]. Although KLF4 has been studied more extensively in ESCs and solid tumors, there is emerging evidence of KLF4 involvement in the process of blood formation by regulating normal hematopoiesis and leukemia stem cells (LSCs).

**Regulation of Self-Renewal by KLFs in Pluripotent Stem Cells**

Because most of what is known on the regulation of self-renewal by KLFs has been described in ESCs, we will briefly review the main contributions before discussing the role of KLF4 in normal and malignant HSCs. ESCs are derived from the inner cell mass of the blastocyst—an early stage of the preimplantation embryo—and characterized for their capacity for self-renewal and pluripotent differentiation of ESCs into any tissue and cell type [24, 25]. ESCs regulate self-renewal and pluripotency properties through cell-intrinsic (NANOG, OCT4, SOX2, and KLFs) and extrinsic factors (leukemia inhibitory factor, LIF) [1, 26–34]. OCT4, SOX2, and NANOG were originally identified as members of a core regulatory pathway involved in the preservation of stemness by promoting self-renewal and preserving pluripotency. Several KLFs (KLF1, KLF2, KLF4, KLF5, and KLF17) regulate self-renewal in the embryos, ESCs, hematopoietic cells, and bone marrow stromal cells in mice, humans, and zebrafish (Table 1). Among these KLFs, the most extensively studied and characterized are KLF2, KLF4, and KLF5, as they form part of a transcriptional circuitry that promotes self-renewal in ESCs by activating pluripotency-associated genes such as NANOG that inhibit their differentiation into primitive endoderm [1, 2]. Abrogation of self-renewal and terminal differentiation of ESCs by simultaneous knockdown of KLF2, KLF4, and KLF5 suggested a KLF regulatory circuitry, which was rescued by the introduction of RNAi-resistant cDNA encoding these three factors [1]. The KLF2/KLF4/KLF5 triad controls self-renewal by regulating the expression of genes involved in self-renewal (Oct4, Sox2, Myc, and Tcl1) and pluripotency (Nanog, Esrrb, and Oct4), facilitating the formation of autoregulatory loops among Oct4, Sox2, Nanog, and Tcl1 in ESCs (Fig. 3) [1, 2]. Although most KLF proteins (KLF1–KLF10) can bind to the regulatory regions of Nanog, only KLF2, KLF4, and KLF5 are able to maintain murine ESCs in an undifferentiated state in the absence of LIF [2]. Based on chromatin immunoprecipitation and sequencing analysis, KLF4 and KLF5 inhibit differentiation of mesoderm and endoderm in ESCs by activating targets other than NANOG [18]. These findings suggest that KLF2, KLF4, and KLF5 have overlapping functions but also exert distinct roles in self-renewal of ESCs. Although KLF4 restores loss

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| KLF proteins | Host organism | Tissue/cell analyzed | Key functionality | Genes regulated by KLFs |
|--------------|---------------|---------------------|------------------|------------------------|
| KLF1         | Mouse         | Embryo [7]          | Required for erythropoiesis [7] | Myc [7]               |
| KLF2         | Mouse         | Embryo and embryonic stem cells [1, 2, 7] | Promotes self-renewal and pluripotency [1, 2] | Nanog and Esrrb [1, 2] |
|              |               |                     | Required for erythropoiesis [7] | Oct4, Tcl1, Nr5a2, Tbx3, Rif1, Sox2, Tcf3, Mycn, and Foxd3 [1] |
|              | Human         | Bone marrow stromal cells [3] | Promotes self-renewal and pluripotency [3] | Oct4, Nanog, and Rex1 [3] |
|              | Zebrafish     | Embryo [6]          | Required for embryogenesis [6] | Oct4 [6]               |
| KLF4         | Mouse         | Embryonic stem cells [1, 2] | Promotes self-renewal and pluripotency [1, 2] | Nanog and Esrrb [1, 2] |
|              |               |                     |                  | Tcl1, Myc, Nr5a2, Tbx3, Nanog, Esrrb, Rif1, Oct4, Sox2, Tcf3, Mycn, and Foxd3 [1] |
| KLF5         | Mouse         | Embryonic stem cells [1, 2, 5] | Promotes self-renewal and pluripotency [1, 2, 5] | Nanog [1, 2, 5] |
|              |               |                     |                  | Esrrb [1, 2] |
|              |               |                     |                  | Oct4 and Sox2 [1, 5] |
|              |               |                     |                  | Tcl1, Myc, Nr5a2, Tbx3, Esrrb, Rif1, Oct4, Tcf3, Mycn, and Foxd3 [1] |
| KLF17        | Zebrafish     | Embryo [6]          | Required for embryogenesis [6] | Oct4 [6]               |

Abbreviation: KLF, Krüppel-like factor.
of stemness caused by deletion of Klf5 in murine ESCs [30], the fact that Klf4-null embryos can develop to term suggests either that KLF4 is dispensable for embryogenesis or there is a functional compensation by other KLF proteins [1, 35]. KLF4 and NANOglob are among the first transcription factors to shut down their transcription when ESCs exit pluripotency, and nuclear export of KLF4 upon ERK activation is a critical first step to exit the naïve pluripotent state and initiate ESC differentiation [16, 36]. In addition, KLF4 is required for expression of the telomerase reverse transcriptase (TERT) in human ESCs and binds β-catenin through protein-to-protein interaction, allowing the recruitment of this dimer to the Tert promoter in murine ESCs [17, 37]. Finally, KLF4 acts as a fast mediator of LIF signaling through the activation of STAT3 to cooperate with OCT4 and SOX2 in activating the expression of NANOglob while repressing the GATA6 and SOX17 genes, which are involved in endoderm differentiation [31]. In summary, several KLF transcription factors regulate key processes of stem cell function in ESCs, among which KLF2, KLF4, and KLF5 play prominent roles. The formation of a KLF circuitry may be exclusive to ESCs, as this mechanism has not been described in other stem cells.

ROLE OF KLF4 IN NORMAL HSCs

Regulation of HSC Self-Renewal

The identification of mechanisms that promote ex vivo self-renewing expansion is the Holy Grail in HSC research and is pursued by many groups aiming at bone marrow transplant and cell and gene therapy applications. The bone marrow milieu modulates stemness at different levels through secreted factors (stem cell factor, thrombopoietin, interleukin-3 [IL3], IL-6, IL-11, and fms-like tyrosine kinase 3 [FLT3]), inflammatory cytokines (e.g., tumor necrosis factor alpha and interferon gamma), hypoxia, the extracellular matrix, and topographic direction of the mitotic spindle with respect to cellular components of the niche during cell division, which could lead to losses of key cellular interactions and an asymmetric distribution of intracellular components. This specialized milieu delivers signals to HSCs through factors recognized by the corresponding receptors that translate information to nuclei, where transcription factors regulate the expression of genes involved in the control of self-renewal. Some of the extrinsic mechanisms regulating HSCs are NOTCH1, hedgehog, WNT, EP receptor for prostaglandin E2, angioptotin-like protein 5, and pleiotrophin (review and references therein [38–41]) (Fig. 1). It is not clear whether KLF4 plays a role in the regulation of these extrinsic mechanisms (Fig. 1), although KLF4 can inhibit the WNT pathway in intestinal epithelium through interaction with β-catenin and repress the expression of NOTCH1 in keratinocytes, whereas NOTCH1 inhibits the expression of KLF4 in intestinal epithelium [42–44].

In addition to the regulation by stem cell niches, many intrinsic factors have been described as positive regulators of HSC self-renewal (e.g., HOXB4, RUNX1, BMI1, p53, mir-126, FLT3, STAT5A, HMGAA2, and SALL4; Fig. 1) [45–52]. Collectively, intrinsic factors regulate cell fate during differentiation at different levels, such as control of gene expression via transcriptional regulation (e.g., RUNX1 and STAT5A), metabolic sensing of nutrients and growth factors (e.g., mTORC1), response to hypoxia and metabolism (e.g., hypoxia-inducible factor 1α), and development and aging (e.g., BMI1 and p16). Reflecting the complexity of self-renewal regulation, many of these factors have interconnected functions; for example, TCF7 regulates the expression of RUNX1 independently of WNT signaling, and the histone H2A deubiquitinaite MYST1 drives the recruitment of RUNX1 into the GFI1 locus, another transcription factor involved in HSC self-renewal [53–55]. Although KLF4 has not been directly associated with the intrinsic regulation of HSC self-renewal, a few reports suggest a potential role in steady-state hematopoiesis, such as the inhibitory effect on BMI1 in intestinal cells, inhibition of mTORC1 during somatic cell reprogramming, and regulation of KLF4 by p53 in acute myeloid leukemia (AML) [56–58]. In addition to transcriptional regulation, the expression of genes involved in self-renewal can be mediated through epigenetic mechanisms such as CpG methylation and histone modifications. For example, mutations in DNMT3A lead to an increase in self-renewal and upregulation of stemness genes in HSCs, and loss of DNMT3A promotes expansion of HSCs in the bone marrow [59–61]. Interestingly, DNMT3A binds to the CpG island in the KLF4 promoter in endothelial cells, inducing DNA methylation and subsequent gene repression [62]. In addition to DNMT3A, IDH1/2 and TET2 mutations, often found in hematologic malignancies, can deregulate the pattern of genomic DNA methylation and aberrantly increase self-renewal [63]. It was recently reported that TET2 binds to KLF4 through protein-to-protein interaction to drive locus-specific demethylation during reprogramming of B cells into iPSCs [64]. Lastly, maintenance of telomere length through telomerase activity also plays a critical role in self-renewal, as loss of telomerase results in reduced self-renewal capacity of HSCs, as evaluated by serial transplantation, in addition to promoting carcinogenesis via genomic instability [65]. Interestingly, KLF4 activates TERT expression through interaction with β-catenin in ESCs [37, 66]. Finally, factors involved in the differentiation toward different lineages, not listed here, could be considered negative regulators of self-renewal because the alternative fate during cell division is differentiation.

KLF4 Regulates Self-Renewal in Adult HSCs

In the hematopoietic system, KLF4 promotes macrophage and monocyte differentiation, macrophage polarization, survival of natural killer cells, secondary antibody responses in memory B cells, and dendritic cell development, whereas KLF4 inhibits homeostatic proliferation of naïve T cells [4, 67–74]. The enrichment of
KLF4 transcripts in human HSCs (CD34+ CD38lo Lin−) compared with HPCs (CD34+ CD38hi Lin−) led to the study of KLF4 in HSCs from fetal livers, because embryonic homozygous deletion results in postnatal lethality [35, 70, 75]. Although clonogenic and competitive transplantation assays of fetal Klf4-null HSCs showed normal colony formation in methylcellulose cultures and hematologic reconstitution of cytoablated recipient mice [70], the role of KLF4 in adult bone marrow HSCs has not been investigated. Further supporting a potential role of KLF4 in adult HSCs, loss of the cell fate determinant lethal giant larvae homolog 1 increases self-renewal, resulting in elevated numbers of HSCs and a competitive advantage after transplantation that is associated with KLF4 repression [41]. This finding suggests that inactivation of KLF4 might contribute to the regulation of self-renewal in adult HSC and warrants the study of KLF4 using somatic gene deletion.

**ROLE OF KLF4 IN LEUKEMIC STEM CELLS**

**KLF4 in Cancer**

KLF4 can behave as a tumor suppressor and an oncogene in a cell context-dependent manner because of its dual properties as activator and repressor of gene expression. For example, KLF4 has a tumor suppressor function in the gastrointestinal tract, whereas in breast cancer KLF4 has oncogenic properties [76, 77]. The potential mechanisms of KLF4 duality in solid-tumor carcinogenesis have been reviewed and depend on multiple factors, such as cell cycle status (expression of p21 and p53), presence of coactivators or corepressors, chromatin accessibility, the epigenome, regulation of oncogenic pathways (e.g., WNT, RAS, TGFβ, and NOTCH1), and interplay with oncogenic drivers [76–79]. In contrast to solid tumors, the role of KLF4 in hematological malignancies has not been well-studied, in large part because in the genomic era, no widespread genetic alterations (mutations, chromosomal translocations) have been found in patients, although epigenetic regulation of tumor suppressors and oncogenes also contributes to the leukemogenic process. At least two inactivating mutations of KLF4 have been identified in childhood acute lymphoblastic leukemia (ALL), both in the 3 prime untranslated region, abolishing a miR-2909 regulatory domain and a zinc-finger motif; the latter inactivates its DNA-binding capacity [80]. However, most of the role of KLF4 in cancer appears to be mediated through epigenetic or post-transcriptional gene inactivation.

Low levels of KLF4 transcript have been detected in AML, B-cell non-Hodgkin and Hodgkin lymphomas, multiple myeloma, and T-cell ALL (T-ALL), suggesting potential tumor-suppressive properties [81–84]. Our group and others found that KLF4 gene expression is silenced by promoter hypermethylation in B-cell lymphomas and T-ALL [81, 82]. In addition to CpG methylation, downregulation of KLF4 is associated with deregulation of micro-RNAs such as miR-10a and miR-10b, and the transcriptional repression of KLF4 by CDX2 in AML is associated with inhibition of PPARγ signaling [84–86]. Conversely, when human CD34+ cells are transduced with ZMYM2-FGFR, the fusion protein product of the t(8;13)(p11;q12) chromosomal translocation found in myeloproliferative neoplasm, and transplanted into NSG mice, the mice display elevated KLF4, suggesting a potential oncogenic function [87]. In contrast, AML patients with a low level of HDAC1, which is negatively correlated with KLF4 level, exhibit better prognosis [88]. More recently, KLF4 expression has been identified in resistant clones in chronic lymphocytic leukemia evaluated by performing single-cell RNA sequencing during diagnosis, treatment, and relapse [89]. Therefore, KLF4 may be involved in the emergence of aggressive leukemic clones during clonal evolution. Collectively, these findings indicate that KLF4 likely regulates the maintenance of LSCs in leukemia.

**KLF4 in Leukemia Stem/Initiating Cells**

LSCs and leukemia-initiating cells (LICs) are rare populations considered leukemia reservoirs, because they can survive chemotherapy and induce relapses and therefore are considered important targets for the development of curative therapies. In stem cell leukemias, LSCs are generated by transformation of a single HSC with a genetic driver mutation that retains the self-renewal property (e.g., chronic myeloid leukemia), whereas LICs are originated by transformation of HPCs that are devoid of self-renewal capacity, and therefore their transformation must be accompanied by acquisition of self-renewal (Fig. 2). The study of the molecular regulation that maintains LSCs/LICs has the main goal of uncovering potential targets for therapy and involves testing the ability of enriched populations to initiate disease after transplantation into secondary wild-type mice, which is the gold-standard assay to evaluate self-renewal capacity.

Research on the role of KLF4 in the maintenance of malignant HSCs has not been actively pursued despite mounting evidence supporting a potential function in the regulation of self-renewal in LSCs or LICs. We recently reported that loss of KLF4 promotes activation of a kinase pathway that drives expansion of LICs in pediatric patients suffering from T-ALL, a lethal blood cancer. Despite significant improvements in the management of children with T-ALL through risk-adaptive therapy, central nervous
KLF4 regulation of self-renewal in ESCs and T-ALL LICs. A KLF self-regulated triad regulates self-renewal in ESCs. In T-ALL LICs, KLF4’s repression of the kinase MAP2K7 is prevented by CpG methylation of the KLF4 promoter. Abbreviations: ESCs, embryonic stem cells; JIP, JNK-interacting protein; KLF, Krüppel-like factor; LIC, leukemia-initiating cell; LIF, leukemia inhibitory factor; Notch1-ic, Notch1 intracellular; MAP2K7, mitogen-activated protein kinase kinase 7; MAP3K, mitogen-activated protein kinase 3; T-ALL, T-cell acute lymphoblastic leukemia.

**Figure 3.**

Despite a vast literature describing the role of KLF4 in the self-renewal of ESCs, the function of KLF4 in normal and malignant HSCs is less well-known. This review provides evidence that KLF4 regulates hematopoiesis and self-renewal of T-ALL LICs through repression of the MAP2K7 pathway and does not involve a KLF circuitry as in ESCs or direct regulation of the cell cycle by KLF4 as seen in solid tumors. A broader knowledge of KLF4 function in blood stem/progenitor cells is necessary to understand the leukemogenic process and how leukemia is driven during treatment and relapses.

**CONCLUDING REMARKS**

System-directed chemotherapy, and supportive care, relapse patients have a poor prognosis, and the rate of complete remission drops significantly in each marrow relapse [90]. The chemoresistance capacity of LICs in addition to their ability to self-renew and induce relapses has made this rare population a target of multiple studies aimed at the development of targeted therapy that is not available yet for T-ALL or any other leukemia [91–95]. Inactivation of KLF4 by somatic gene deletion in mouse models accelerates the development of NOTCH1-induced leukemia by enhancing the G1-to-S transition in leukemic cells and promoting the expansion of LICs (Fig. 3) [96]. An analysis of global gene expression and genome-wide binding of KLF4 in murine T-ALL cells revealed that KLF4 represses the gene encoding the dual-specificity mitogen-activated protein kinase kinase 7 (MAP2K7) (Fig. 3). In contrast to ESCs, a network of KLF proteins was not identified in T-ALL LICs. MAP2K7 is part of a three-tiered signaling unit consisting of upstream MAP3K and downstream MAPK, held together by a scaffolding protein known as c-Jun N-terminal kinases (JNK)-interacting protein, and although the kinase upstream of MAP2K7 has not been identified, particularly in leukemic T cells, the only known downstream substrate of MAP2K7 is JNK [97]. JNK activation in turn phosphorylates the final effectors of this pathway, ATF2 and c-Jun, which are involved in the control of cellular proliferation and believed to fuel expansion of LICs. Strikingly, patients exhibit low levels of KLF4 that was associated with hypermethylation of the KLF4 promoter and aberrant activation of the MAP2K7 pathway, similar to T-ALL mouse deficient in KLF4, because normally KLF4 represses the MAP2K7 gene [96]. The inactivation of KLF4 across pediatric T-ALL patients and the frequency of NOTCH1 mutations (approximately 50%) suggest that MAP2K7 activation is likely independent of the driver mutation used to induce T-ALL in mice. The fact that deletion of KLF4 results in upregulation of both total and phosphorylated MAP2K7 led to the hypothesis that either leukemic cells exhibit basal activation of this pathway or KLF4 somehow additionally represses MAP2K7 activation. Because KLF4-deficient T-ALL mice show an increased frequency of LICs, defined as CD4−CD8−CD25+IL7Rxα and CD25+ c-Myc+ leukemic cells, which was confirmed by limiting-dose transplantation of leukemic bone marrow cells, it is possible that inhibition of the MAP2K7 pathway can target the LIC population in high-risk T-ALL patients. As a proof of concept, pharmacological inhibition of JNK in T-ALL cells with the CC401 and AS602801 compounds showed significant reduction in the leukemia burden in cell line-based xenograft and patient-derived xenograft models [96]. However, JNK inhibitors show low potency, with antileukemic properties in the micromolar range, and thus one current focus is on the identification of more potent and specific MAP2K7 inhibitors.

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**AUTHOR CONTRIBUTIONS**

C.S.P., A.L., T.C.: manuscript writing, table preparation; D.L.: conception/design, figure design, manuscript writing, final approval of manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicated no potential conflicts of interest.
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