RED CELLS, IRON, AND ERYTHROPOIESIS

RUNX1 and *NF-E2* upregulation is not specific for MPNs, but is seen in polycythemic disorders with augmented HIF signaling

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Key Points

- Overexpression of *RUNX1* and its target *NF-E2* is not specific for PV but is also seen in polycythemias due to augmented hypoxia sensing.
- Elevated levels of *RUNX1* and *NF-E2* are not specific for primary polycythemias, as these are not present in PFCP.

Overexpression of transcription factors runt-related transcription factor 1 (*RUNX1*) and nuclear factor, erythroid-derived 2 (*NF-E2*) was reported in granulocytes of patients with polycythemia vera and other myeloproliferative neoplasms (MPNs). Further, a transgenic mouse overexpressing the *NF-E2* transgene was reported to be a model of MPN. We hypothesized that increased transcripts of *RUNX1* and *NF-E2* might characterize other polycythemic states with primary polycythemic features, that is, those with exaggerated erythropoiesis due to augmented erythropoietin (EPO) sensitivity. We tested the expression of *RUNX1* and *NF-E2* in polycythemic patients of diverse phenotypes and molecular causes. We report that *RUNX1* and *NF-E2* overexpression is not specific for MPN; these transcripts were also significantly elevated in polycythemias with augmented hypoxia-inducible factor activity whose erythroid progenitors were hypersensitive to EPO. *RUNX1* and *NF-E2* overexpression was not detected in patients with EPO receptor (EPOR) gain-of-function, suggesting distinct mechanisms by which erythroid progenitors in polycythemias with defects of hypoxia sensing and EPOR mutations exert their EPO hypersensitivity. (*Blood*. 2014;123(3):391-394)

Introduction

Transcription factor runt-related transcription factor 1 (RUNX1, also known as AML1) is the principal regulator of mammalian hematopoiesis. Aberrant *RUNX1* expression in the hematopoietic lineage, generated by multiple mechanisms (translocations, gain-of-function mutations, and gene amplification), is thought to be causative of leukemic transformation.[1] However, mutations of *RUNX1* are rare in chronic myeloproliferative neoplasms (MPNs).[2]

Transcription factor nuclear factor, erythroid-derived 2 (NF-E2) is a target of RUNX1 that is essential for the regulation of erythroid and megakaryocytic maturation and differentiation and expression of globin genes.[3]

It has been reported that increased *RUNX1* expression in granulocytes is present in all 3 classical MPNs, that is, polycythemia vera (PV), essential thrombocythemia, and primary myelofibrosis. It has been suggested to be specific for MPN,[4] and that elevated NF-E2 promotes erythropoietin (EPO)-independent erythroid maturation of PV hematopoietic stem cells in vitro.[5,6] A mouse model overexpressing the *NF-E2* transgene in hematopoietic cells was reported to be a new model of MPN.[7]

Polycythemic states can be divided into primary polycythemias, characterized by intrinsically hyperproliferative erythroid progenitors that are hypersensitive to EPO, and secondary polycythemias, wherein erythroid progenitors respond normally to EPO but circulating EPO is elevated or inappropriately normal for the level of increased red cell mass.[8,9] Examples of primary polycythemias are PV, gain-of-function mutations of the EPO receptor causing a phenotype of primary familial and congenital polycythemia (PFCP), and some congenital disorders of hypoxia sensing that may share features of both primary and secondary polycythemias, as exemplified by Chuvash polycythemia.[10] In this report, we examined the possibility that increased transcripts of *RUNX1* and *NF-E2* may also be present in other primary polycythemic states.

Study design

Sample processing

We prospectively recruited 26 subjects with various primary and secondary polycythemias (Table 1) using approved University of Utah (23 subjects) and Palacky University Hospital (3 subjects) Institutional Review Board informed consent in accordance with the Declaration of Helsinki. Patients' granulocytes and mononuclear cells were separated from peripheral blood, as previously described.[11] Mouse embryos and yolk sacs were analyzed as described.[12]

In vitro sensitivity assay of erythroid progenitors to EPO

Mononuclear cells were isolated from peripheral blood and subjected to in vitro colony-forming assay, as previously described.[13] Erythroid

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Table 1. Polycythemias studied

	Diagnosis	Gene mutations	Number of patients	Sensitivity of erythroid colonies to EPO
Primary polycythemias	PV	*JAK2*^{V617F} mutation	n = 6	Hypersensitive and EPO independent
	PFCP	*EPOR*^{Q434X} mutation*	n = 2	Hypersensitive and EPO independent
		EPOR^{5967insT} mutation†	n = 2	Hypersensitive and EPO independent
Polycythemias with features of primary and secondary polycythemia	Congenital polycythemias due to *VHL* mutations	*VHL*^{R200W} mutation[10] (Chuvash polycythemia)	n = 2	Hypersensitive
		VHL^{P138L} mutation[16]	n = 1	Hypersensitive
		VHL$^{T124A/L188V}$ mutation[17]	n = 2	Hypersensitive
	Congenital polycythemias due to *HIF2A* mutation	*HIF2A*^{M353V} mutation[18]	n = 1	Hypersensitive
		HIF2A^{G537R} mutation[18]	n = 1	Hypersensitive
	Congenital polycythemia	*LNK*^{I257T} mutation‡	n = 1	Hypersensitive
Secondary polycythemias	Congenital polycythemia due to *VHL* mutation	*VHL*^{H191D} mutation[19] (Croatian type)	n = 2	Normal
	Secondary polycythemia due to hypoxia	Low pO_2	n = 6	Normal

*Two previously unreported subjects of European descent with PFCP due to the *EPOR* gain-of-function *EPOR*^{Q434X} mutation; this mutation was previously reported in a Japanese family.[14]

†Two Czech patients whose phenotype was previously described.[15]

‡A patient with no detectable *EPOR*, *JAK2*^{V617F}, or *JAK2* exon 12 mutations and low level of EPO (<1 mU/mL) who was heterozygous for a single-nucleotide polymorphism in exon 3 (rs147341899) in the *LNK* gene.

burst-forming unit colonies (BFU-Es) were scored by standard morphologic criteria.

Real-time polymerase chain reaction assay

Total RNA was isolated using TRI-reagent (Molecular Research Center, Cincinnati, OH) and treated with DNA-free DNase Treatment and Removal Reagents (Ambion, Life Technologies, NY). DNA-free RNA was reverse-transcribed using a SuperScript VILO cDNA Synthesis Kit (Invitrogen/Life Technologies, NY) according to the manufacturer's instructions and used for quantitative real-time polymerase chain reaction as described.[16]

Results and discussion

The phenotypes and causative mutations of 26 polycythemic patients are depicted in Table 1. All primary polycythemic patients had erythroid progenitor hypersensitivity to or independent of EPO (Figure 1A); all secondary polycythemic subjects had normal BFU-E EPO sensitivity (data not shown). To assess whether the putative mechanism underlying the intrinsic hypersensitivity of erythroid progenitors to EPO in PV[5,6] is unique to PV or shared with other polycythemia states, we analyzed *RUNX1* and *NF-E2* expression in hypersensitive BFU-Es.

Elevated *RUNX1* and *NF-E2* gene transcripts in hypersensitive BFU-Es and granulocytes from patients with PV and polycythemias with defects of hypoxia sensing

All examined PV patients, polycythemia patients with defects of hypoxia sensing (2 unrelated subjects with Chuvash polycythemia, 1 polycythemic patient homozygous for the *VHL*^{P138L} mutation,[16] and 1 patient with the *HIF2A*^{M535V} gain-of-function mutation[18]), and 1 patient with a heterozygous single-nucleotide polymorphism in the *LNK* gene (rs147341899; *LNK*^{I257T}) had elevated *RUNX1* and *NF-E2* gene transcripts in their BFU-Es (Figure 1A). *RUNX1* and *NF-E2* gene transcripts were also increased in granulocytes of these patients and in granulocytes of another gain-of-function *HIF2A* mutant (*HIF2A*^{G537R}) patient[18] from whom RNA from BFU-Es was unavailable (Figure 1B).

Patients with PFCP do not have elevated *RUNX1* and *NF-E2* gene transcripts

We tested whether increased *RUNX1* and *NF-E2* gene transcripts characterize all primary polycythemias. PFCP-derived BFU-Es and/or granulocytes did not have increased levels of these transcripts in cells with the *EPOR*^{Q434X} mutation[14] nor in BFU-Es from PFCP patients with *EPOR*^{5967insT} mutation[15] (granulocytes were unavailable) (Figure 1A-B).

Some disorders of hypoxia sensing have elevated *RUNX1* but not *NF-E2* gene transcripts

We next examined granulocytes from 2 Croatian polycythemic patients with a homozygous *VHL*^{H191D} exon 3 gene mutation whose erythroid progenitors were not hypersensitive to EPO[19] and found *RUNX1* transcripts, but not *NF-E2* transcripts, increased (Figure 1B). We observed similar results in 2 compound heterozygotes for *VHL*^{T124A} and *VHL*^{L188V} mutations. These 2 polycythemic siblings had hypersensitive erythroid colonies[17] and increased *RUNX1*, but not *NF-E2*, transcripts in granulocytes (Figure 1B). RNA from their BFU-Es was unavailable for testing.

Secondary polycythemia had normal *RUNX1* and *NF-E2* transcripts

All 6 unrelated subjects with secondary polycythemia had normal *RUNX1* and *NF-E2* gene transcripts in granulocytes (Figure 1B).

Patients with increased *RUNX1* gene transcripts have increased transcripts of HIF targets

We then examined granulocyte transcripts of the hypoxia-inducible factor (HIF)-regulated genes *TFRC*, *SLC2A1*, *HK1*, *PDK1*, *VEGF*, and *BNIP3* and found them to be increased in all PV patients and all studied polycythemic patients with increased *RUNX1* gene transcripts, but not in polycythemic *EPOR*^{Q434X} patients or 6 patients with secondary polycythemia (Figure 1B). *EPOR*^{5967insT} granulocytes were unavailable.

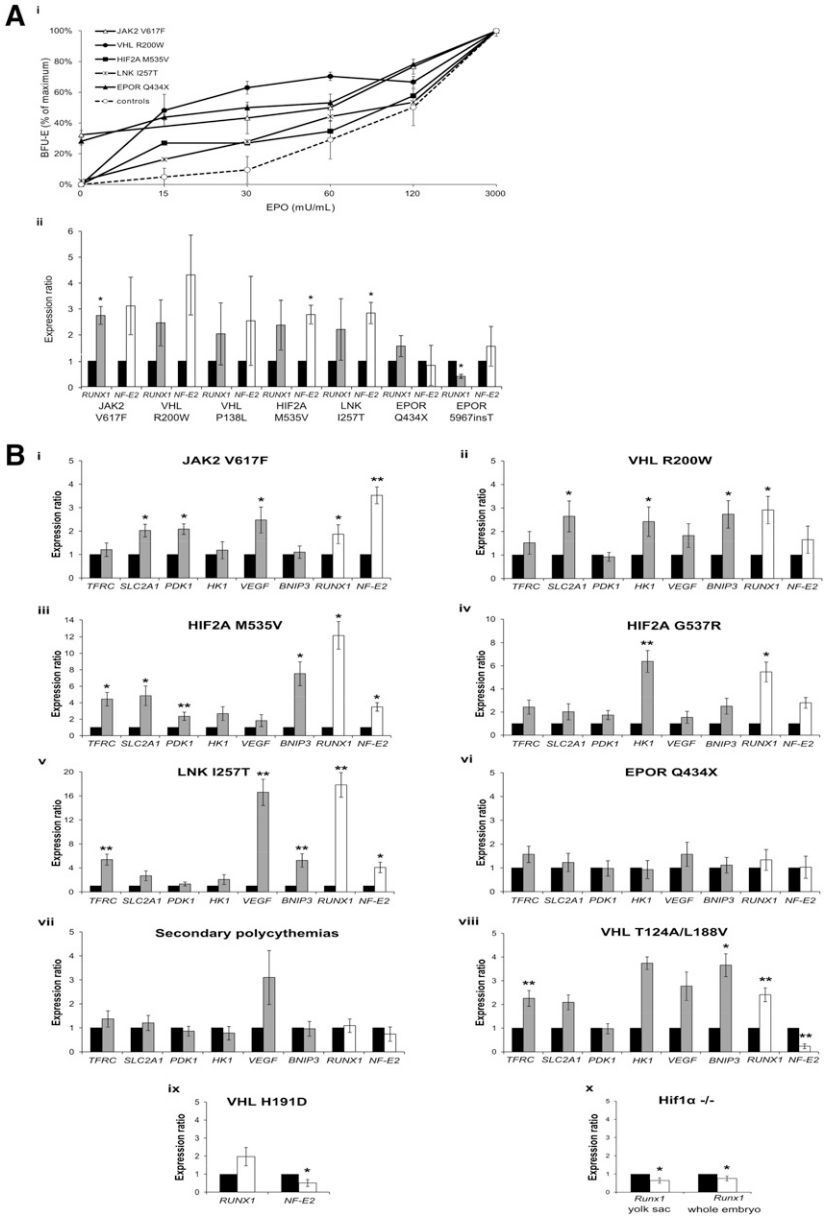

Figure 1. (A) (i) Sensitivity of BFU-E erythroid progenitors to EPO. Hypersensitive EPO response characterized by the increased in vitro growth of BFU-Es in the presence of low concentrations of EPO (0-30 mU/mL) was found in all patients with *JAK2*^{V617F} (n = 3), *EPOR*^{Q434X} (n = 2), *HIF2A*^{M535V} (n = 1), *VHL*^{R200W} (n = 2), and *LNK*^{I257T} (n = 1) mutations. In patients with *JAK2*^{V617F} and *EPOR*^{Q434X} mutations, some BFU-E colonies also grew in the absence of EPO. Dashed line show the response of erythroid progenitors to EPO of tested healthy controls (n = 9). The number of BFU-Es grown in individual concentrations of EPO was expressed as a percentage of maximum vs the concentration of EPO. Results were pooled when n > 1. T bars designate standard deviations. **(ii) Relative expression of *RUNX1* and *NF-E2* in hypersensitive BFU-E colonies.** BFU-Es grown in low concentrations of EPO (15-30 mU/mL, ie, EPO-hypersensitive BFU-Es) were harvested and used for expression assay. Expression in BFU-Es was analyzed from patients with *JAK2*^{V617F} (n = 3), *VHL*^{R200W} (n = 2), *VHL*^{P138L} (n = 1), *HIF2A*^{M535V} (n = 1), *LNK*^{I257T} (n = 1), *EPOR*^{Q434X} (n = 2), and *EPOR*^{5967insT} (n = 2) mutations. The *RUNX1* (Hs00257856) and *NF-E2* (Hs00232351) TaqMan Gene Expression probes were used for quantitative real-time polymerase chain reaction. All samples were investigated in triplicate and normalized to expression of *HPRT* (4333768F) and *GAPDH* (4333764F) reference genes. The data were normalized to mRNA levels of healthy controls (black, n = 6), T bars designate SEM; *$P < .05$. The statistical significance of relative expression changes in target mRNA levels were analyzed for all expression analysis using REST 2009 software.[20] **(B) (i-ix) Relative expression of *RUNX1*, *NF-E2*, and HIF-regulated genes in granulocytes.** Expression in granulocytes was analyzed from patients with *JAK2*^{V617F} (n = 6), *EPOR*^{Q434X} (n = 2), *HIF2A*^{M535V} (n = 1), *HIF2A*^{G537R} (n = 1), *VHL*^{R200W} (n = 2), *LNK*^{I257T} (n = 1), *VHL*$^{T124A/L188V}$ (n = 2), and *VHL*^{H191D} (n = 2) mutations and patients with secondary polycythemia (n = 6). The following TaqMan Gene Expression probes were used for quantitative real-time polymerase chain reaction: *transferrin receptor* (*TFRC*; Hs00951083), *glucose transporter-1* (*SLC2A1*; Hs00892681), *vascular endothelial growth factor* (*VEGF*; Hs00900055), *BNIP3* (Hs00969291), *hexokinase-1* (*HK1*; Hs00175976), *pyruvate dehydrogenase kinase, isozyme 1* (*PDK1*; Hs01561850), *RUNX1* (Hs00231079), and *NF-E2* (Hs00232351). All samples were investigated in triplicate and normalized to expression of *HPRT* (4333768F) and *GAPDH* (4333764F) reference genes. The data represents the mean of 3 independent experiments and were normalized to mRNA levels of healthy controls (black, n = 16); T bars designate SEM; *$P < .05$ and **$P < .01$. **(x) Relative expression of *Runx1* in *Hif1α*$^{-/-}$ yolk sacs and whole embryos.** Expression in samples isolated from *Hif1α*$^{-/-}$ yolk sacs (n = 7) and whole embryos (n = 6) were analyzed using TaqMan Gene Expression probe for mouse *Runx1* gene (Mm0123404). The data were normalized to expression of β-actin (*Actb*; 4352341E) and to mRNA levels of stage-matched, wild-type yolk sacs (black, n = 7) and whole embryos (black, n = 6); T bars designate SEM; *$P < .05$.

Regulation of *Runx1* transcript in *Hif1α*$^{-/-}$ mouse embryo and yolk sac

To further support our hypothesis that HIF signaling regulates *RUNX1* expression, we analyzed *Hif1α*$^{-/-}$ whole mouse embryo (*Hif1α* deficiency is embryonic lethal by day 11) and hematopoietic tissue, ie, murine *Hif1α*$^{-/-}$ yolk sac (E9.5).[12] The *Runx1* transcript was down-regulated (Figure 1Bx), confirming that HIF directly or indirectly regulates RUNX1.

In summary, we found increased expression of *RUNX1* in all patients with augmented HIF signaling, including PV patients. Further, in some, but not all, patients with augmented HIF signaling, we also detected elevated *NF-E2* transcripts. Hypersensitive erythroid progenitors derived from patients with PV and augmented HIF signaling, but not PFCP, share elevated expression of *RUNX1* and *NF-E2*. This suggests that HIF-mediated mechanisms, by which erythroid progenitors in PV and polycythemias with augmented hypoxia sensing, may exert their EPO hypersensitivity by up-regulation of *RUNX1* and *NF-E2*. However, this is not a mechanism of erythroid EPO hypersensitivity in *PFCP*. The augmented HIF signaling in PV has not been well described; however, in our preliminary report, we observed increased HIF activity, the so-called "Warburg effect," in PV patients.[21] We conclude that increased expression of *RUNX1* and *NF-E2* is not specific for PV and other MPNs and is not universal for all primary polycythemic disorders such as PFCP, but it is present in those primary polycythemias with augmented HIF signaling.

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Authorship

Contribution: K.K. performed the research, analyzed data, and wrote the paper; L.L. performed some research, analyzed data, and wrote the paper; F.L. performed some research and reviewed the paper; J.S. prepared and purified RNA from *Hif1α*$^{-/-}$ mice; M.H. contributed to the research and reviewed the paper; V.D. wrote the paper and provided financial support; and J.T.P. conceived the study, wrote the paper, and provided financial support.

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