ORIGINAL ARTICLE

Identification of Hepatic-like EPO as a Cause of Polycythemia

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ABSTRACT

BACKGROUND

Secondary erythrocytosis often results from conditions that cause tissue hypoxia or an improper increase in erythropoietin (EPO) production. EPO, the major regulator of erythropoiesis, has a complex and tightly regulated expression during development, with a liver-to-kidney switch shortly after birth.

METHODS

We identified six families with erythrocytosis that was associated with circulating EPO levels within the normal range and characterized as a novel molecular and functional entity. We investigated the effect of the identified pathogenic variants using *EPO* promoter–driven luciferase reporter genes. Induced pluripotent stem cells (iPSCs) were generated from patient cells and differentiated into hepatocyte-like EPO-producing cells. Samples of circulating EPO from patients with hereditary erythrocytosis and from healthy newborns were analyzed by means of isoelectric focusing, and EPO activity was assessed.

RESULTS

Three novel variants were identified in the noncoding regions of *EPO*. Experiments with reporter assays and iPSC-derived hepatocyte-like cells showed that the variants targeted previously uncharacterized regulatory elements of the gene, which, when the variants were present, showed high responsiveness to hypoxia. EPO samples from all the patients showed a modified isoelectric-focusing profile, identical to hepatic EPO that is expressed in premature neonates and in patients with acquired erythrocytosis associated with liver diseases. EPO that was purified from patient plasma and umbilical-cord blood samples showed enhanced EPO receptor signaling activity in vitro, which suggests a potential gain of function linked to the liver-type glycosylation of EPO.

CONCLUSIONS

We found that secondary erythrocytosis can be related to variants in *EPO* that lead to the production of hepatic-like EPO with an atypical glycosylation pattern and increased activity. (Funded by Région des Pays de la Loire and others; ClinicalTrials.gov number, NCT03957863.)

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Erythropoietin (EPO), a glycoprotein hormone, is a fundamental regulator of erythropoiesis. The expression of *EPO* is tightly regulated by local changes in the partial pressure of oxygen. When oxygen levels decrease, cells trigger the stabilization of hypoxia-inducible factor (HIF), which in turn initiates HIF-dependent transcriptional induction of *EPO* expression.[1-5] During development, EPO is expressed by neural crest cells and fetal hepatocytes. At birth, expression in the liver decreases, and the interstitial cells of the kidney, which originate from the neural crest cells, become the principal site of production.[2,6] In adults, EPO is produced predominantly (90%) by the kidney and, to a lesser extent, by other organs, particularly the liver.[2,7]

Pathogenic variants in genes of the oxygen-sensing pathway have been identified in patients with hereditary erythrocytosis, a rare hematologic disease characterized by the excessive production of red cells.[8] These genes include *VHL* (which encodes von Hippel–Lindau tumor suppressor),[9] *EGLN1* (also called *PHD2*, which encodes prolyl hydroxylase domain–containing protein 2),[10,11] *EPAS1* (also called *HIF2A*, which encodes HIF-2α),[12,13] and *EPO*.[14,15] However, the exact molecular mechanism underlying the overproduction of red cells in patients with *EPO* variants is still enigmatic owing to discrepancies among high EPO messenger RNA (mRNA) levels in the mutant cellular models, serum EPO levels that are frequently within the normal range, and increased red-cell mass in patients.[15]

In this study, we investigated variants in the noncoding regions of the *EPO* locus in six families. We studied the effects of these variants using reporter assays and patient-derived models of induced pluripotent stem cells (iPSCs) differentiated into hepatocyte-like cells. The profile of patients' circulating EPO was characterized by means of isoelectric focusing[16] and compared with profiles of samples obtained from healthy adults, patients with secondary erythrocytosis, and premature newborns, whose expression of EPO is controlled by the liver. The activity of these different EPOs purified from blood was also evaluated. Our aim was to explain puzzling observations in patients with unexplained erythrocytosis.

METHODS

SAMPLES FROM PATIENTS

All the study participants (or the parents or guardians of participants who were minors) provided written informed consent. Blood samples were obtained for diagnostic and research purposes after the approval of the study by the local ethics committee. Samples that had been obtained from newborns and children were provided by Centre Hospitalier Universitaire of Dijon, France. Cord-blood samples were provided by the Biologic Resources Center of Hôpital Saint-Louis, Assistance Publique–Hôpitaux de Paris, Paris. After blood centrifugation was performed, plasma samples were kept frozen at –20°C until analysis. For DNA sequencing, DNA was extracted from whole blood, and molecular screening was performed by means of high-throughput sequencing with a sequencing panel containing hypoxia-related pathway genes. The classification of genetic variants was guided by criteria from the American College of Medical Genetics and Genomics (ACMG). Details of these criteria are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

FUNCTIONAL ANALYSES

Luciferase reporter gene assays were performed with the use of human embryonic kidney (HEK293T) cells and Hep3B cells (a human hepatocarcinoma cell line that expresses hypoxia-inducible *EPO*) transfected with wild-type and mutated constructs containing proximal and distal *EPO* regulatory elements.[17] Constructs were generated by site-directed mutagenesis, canonical restriction enzyme–based cloning, and In-Fusion cloning, with variants confirmed by Sanger sequencing. Cell culture experiments were performed under normoxic and hypoxic conditions (24 hours at 0.2% oxygen). Human iPSCs were derived from patient cells and subsequently differentiated into hepatocyte-like cells.[18,19] Hepatic markers and *EPO* expression were analyzed by means of reverse-transcriptase–quantitative polymerase-chain-reaction testing. Plasma EPO profiles were analyzed by means of isoelectric-focusing analysis.[20] EPO purification was performed by immunoprecipitation, and EPO activity was measured with the use of an AlphaLISA SureFire Ultra p-STAT5 assay in UT-7/EPO cells (a human leukemia cell line that expresses the EPO receptor and is EPO-dependent). Further details of the methods are provided in the Supplementary Appendix.

STATISTICAL ANALYSIS

Data are presented as means with the standard error of the mean. Differences in means among

multiple groups were analyzed with the use of a one-way analysis of variance or, when not applicable, with Kruskal–Wallis tests followed by Tukey or Dunn post hoc tests. Pathogenic variant and concentration effects were analyzed with the use of a two-way analysis of variance to account for possible interaction. The effect at each concentration was tested by multiple Tukey post hoc tests. P values of 0.05 or less were considered to indicate statistical significance. The statistical analyses were performed with the use of Prism software, version 9.5.1 (GraphPad).

RESULTS

IDENTIFICATION OF NOVEL VARIANTS IN NONCODING REGIONS OF *EPO*

Patients with erythrocytosis were selected for this study after classic causes of erythrocytosis (polycythemia vera or secondary erythrocytosis associated with certain renal, cardiac, or pulmonary disorders) were ruled out according to a published flowchart.[11] Genetic screening was performed on genomic DNA with the use of a sequencing panel that was specifically designed for erythrocytosis and included genes involved in the hypoxia pathway (*VHL*, *PHD2*, *HIF2A*, and *EPO*). Three different genetic variants were identified in noncoding sequences of *EPO* in six families: the c.–252C→T variant located in the main promoter and the c.14–28T→C and c.14–26A→G variants located in intron 1 in the proximity of exon 2 (Fig. 1).

A history of erythrocytosis was described in 29 patients. The patients who were included in the study presented with erythrocytosis associated with moderate-to-severe elevation in hematologic values (Table 1). Genetic sequencing identified a germline heterozygous variant in all the patients but one (Patient IV-6 in Family F1), who had severe disease and was homozygous for the c.–252C→T variant (Fig. 1B). All the patients had a serum EPO concentration within the normal range (5 to 25 IU per liter). The variants were absent from the Genome Aggregation Database (gnomAD, version 4), they segregated with the disease within the families, and they were detected in different families, which provided strong arguments for their involvement in the pathologic condition. To identify the molecular mechanisms at the origin of developed erythrocytosis, the effect of these novel variants on EPO function was investigated.

FUNCTIONAL STUDIES OF *EPO* VARIANTS

The c.–252C→T variant was located in the main promoter (P1) in close proximity to a region containing binding sites of the transcription factors GATA, HIF, and WT1 (Wilms' tumor 1) (Fig. S1 in the Supplementary Appendix). Luciferase reporter constructs containing the proximal region of the *EPO* promoter in the presence or absence of the distal 5′-HRE (hypoxia response element), 3′-HRE, or both were used to measure the effect of the variant on *EPO* transcription (Fig. 2A). Luciferase assays in HEK293 cells showed significantly increased reporter gene expression with the c.–252C→T mutated promoter, minimal version (EPO Prom), and extended version (Full Prom) under hypoxic conditions for all constructs in the presence of 5′-HRE or 3′-HRE (Fig. 2B and Fig. S2E and S2H and Fig. S5). In a finding that was consistent with results obtained under hypoxic conditions, overexpression of HIF-2α, the major HIF-α isoform regulating *EPO* expression, resulted in increased *EPO* promoter–driven luciferase activity on the c.–252C→T variant (Fig. 2C and Fig. S2I and S2K). Similar results were obtained in Hep3B cells as an independent cell model (Figs. S3 and S6). No changes were observed in the absence of the remote enhancer elements, which suggests that the consequences of promoter c.–252C→T variant involve cooperation with distal regulatory elements (Fig. 2B).

Because the variant was located close to an established GATA binding site, we next explored the potential GATA factor-dependent regulation of luciferase activity in transient transfection experiments. The c.–252C→T variant greatly increased the ability of GATA4 to induce *EPO* expression, without affecting GATA2 and GATA3 inhibitory activity, under normoxic and hypoxic conditions in HEK293 cells (Fig. 2C and Fig. S2M) and Hep3B cells (Fig. S4).

With regard to the functional effect of the variants located in intron 1, upstream of exon 2 (c.14–28T→C and c.14–26A→G), we initially explored two possible mechanisms: the modification of EPO isoforms expressed from the second promoter, P2 (as has been described for the variant c.32delG[15]) (Fig. S7), and the deregulation of exon 2 splicing (Fig. S8). However, we were unable to reach a definitive conclusion regarding the first hypothesis, and we did not identify a common mechanism linking the two variants in relation to the second hypothesis. Consequently, we explored

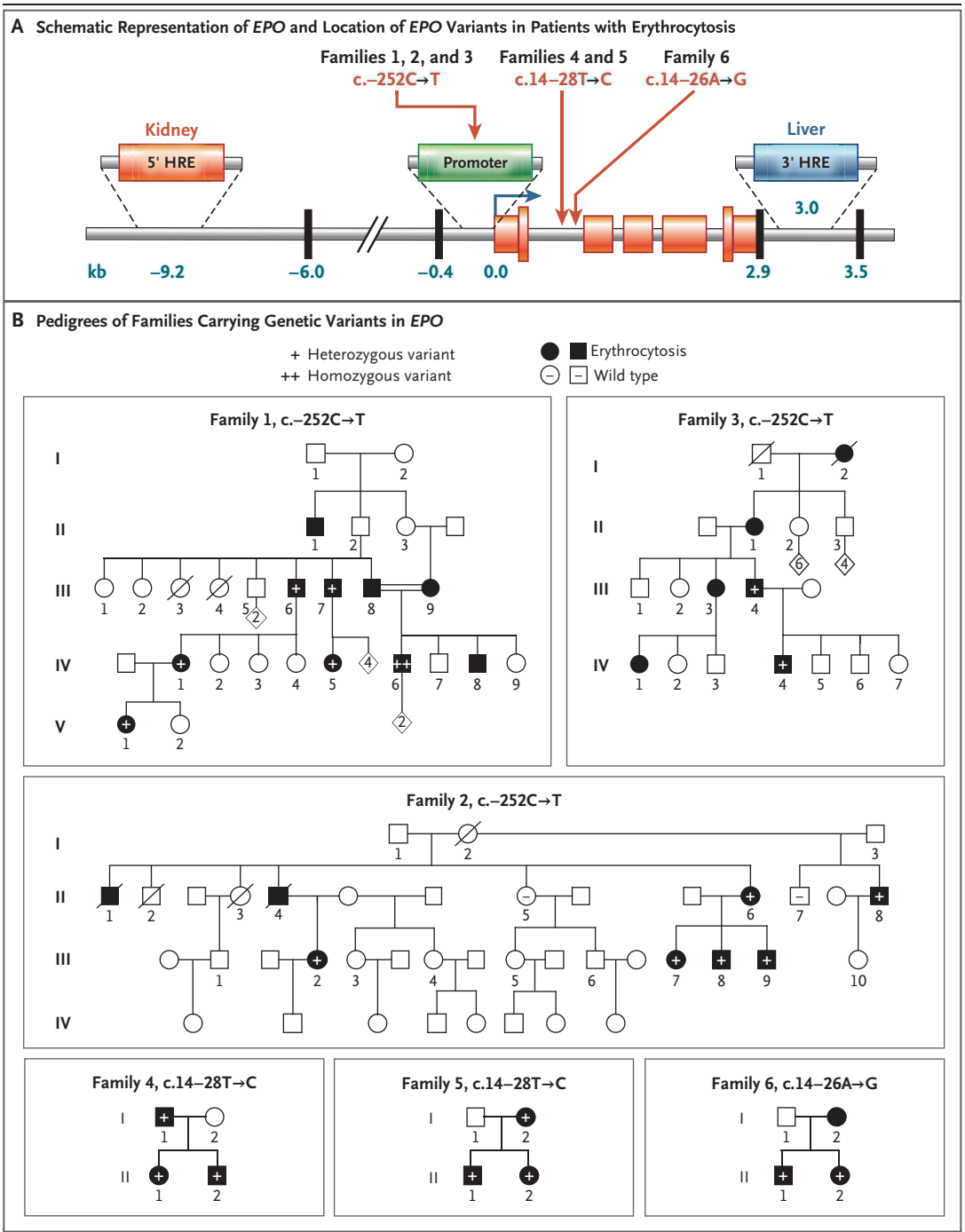


Figure 1. Location and Segregation of *EPO* Variants.

Panel A shows a schematic representation of *EPO*, the gene encoding erythropoietin (EPO) (National Center for Biotechnology Information reference sequence, NM_000799.4), and the location of *EPO* variants identified in patients with erythrocytosis. Panel B shows the pedigrees of six families carrying a genetic variant in *EPO*. Results of genetic testing (indicated by one or two plus signs or the minus sign) are indicated in the key. Circles denote female family members, and squares male family members; a line through a symbol indicates that the person is deceased. Diamonds show the number of children born to a person, without specification of their sex. Patient numbers are shown only for patients and their relatives who were likely to have transmitted the variant; persons outside the family who married into the group were not considered.

Table 1. Clinical Data of the Patients.*

Family No., cDNA Variant, and Patient No.	Genetic Status	Sex	Age at Diagnosis	Current Age	Hematocrit†	Hemoglobin‡	Red Cells§	EPO¶
			yr		*%*	*g/dl*	*×10^{-12} cells/liter*	*IU/liter*
F1, c.–252C→T								
III-6	Heterozygous	M	50	67	51.0	18.2	—	14.5
III-7	Heterozygous	M	34	70	63.0	22.8	6.7	—
III-9	NA	F	49	75	54.0	18.7	—	13.7
IV-1	Heterozygous	F	38	40	41.0	13.4	4.6	5.9
IV-5	Heterozygous	F	18	36	56.0	18.6	6.5	11.0
IV-6	Homozygous	M	21	47	68.0	22.0	—	27.0
IV-8	NA	M	11	37	58.0	18.7	6.4	—
V-1	Heterozygous	F	9	10	53.0	18.0	6.3	3.4
F2, c.–252C→T								
II-5	Wild type	F	—	80	43.2	14.1	5.0	7.6
II-6	Heterozygous	F	60	76	54.0	17.7	6.4	9.6
II-7	Wild type	M	73	74	47.9	16.1	5.6	8.9
II-8	Heterozygous	M	18	67	55.0	17.8	5.4	19.8
III-2	Heterozygous	F	42	58	54.1	18.1	6.6	7.3
III-7	Heterozygous	M	24	41	63.0	20.8	7.3	9.2
III-8	Heterozygous	F	46	49	55.0	18.1	5.9	—
III-9	Heterozygous	M	20	44	55.8	18.2	6.2	6.6
III-10	NA	M	8	9	46.0	15.4	5.7	—
F3, c.–252C→T								
III-3	NA	F	—	49	52.0	18.4	—	—
III-4	Heterozygous	M	—	52	47.8	16.5	5.8	—
IV-1	NA	F	—	25	50.4	17.4	6.3	9.5
IV-4	Heterozygous	M	—	16	66.1	21.3	8.3	5.7
F4, c.14–28T→C								
I-1	Heterozygous	M	33	55	57.0	18.8	6.2	19.0
II-1	Heterozygous	F	22	25	49.0	16.4	5.6	10.0
II-2	Heterozygous	M	13	21	57.0	19.1	6.4	10.0
F5, c.14–28T→C								
I-2	Heterozygous	F	32	78	55.6	19.4	—	19.8
II-1	Heterozygous	M	55	57	52.0	18.0	5.8	6.0
II-2	Heterozygous	F	23	47	50.0	17.3	5.6	—
F6, c.14–26A→G								
II-1	Heterozygous	M	15	26	58.6	20.6	6.5	<10.0
II-2	Heterozygous	F	14	23	66.2	22.6	7.3	14.0

* Shown are the genetic variants identified in *EPO*, the gene encoding erythropoietin (EPO), in patients with erythrocytosis. The term cDNA denotes complementary DNA, F female, M male, and NA not available.
† Normal ranges for hematocrit are as follows: 40 to 52% in men and 37 to 47% in women.
‡ Normal ranges for hemoglobin are as follows: 13 to 17 g per deciliter in men and 12 to 16 g per deciliter in women.
§ Normal ranges for red cells are as follows: 4.2×10^{12} to 5.7×10^{12} per liter in men and 4.2×10^{12} to 5.2×10^{12} per liter in women.
¶ The normal range for EPO is 5 to 25 IU per liter. For Patient II-1 in Family 6, the laboratory did not report a specific value, only that the value was less than 10.0 IU per liter.

a third hypothesis involving transcriptional dysregulation induced by these variants.

Computer-simulation analyses suggested the presence of GATA-binding motifs in the intronic region that might be affected by the variants (Fig. S10A, S10B, and S10C). To investigate this third assumption, we generated a luciferase reporter construct driven by the *EPO* regulatory elements including a partial sequence of the intronic region (Fig. 2A). Then, we separately introduced the c.14–26A→G and c.14–28T→C variants in this novel construct (Fig. 2A and Fig. S9A). Luciferase reporter assays showed that the intronic region contained potential inhibitory regulatory elements that reduced the expression of the reporter gene under hypoxic conditions (Fig. 2D).

We next evaluated the effect of the c.14–26A→G and c.14–28T→C variants in the presence of coexpressed GATA factors that are known to be involved in *EPO* regulation. Transient transfection experiments revealed that the introduction of the variants in these reporter constructs further counteracted this decreased *EPO* expression by altering the effects of GATA2 (c.14–28T→C), GATA3 (c.14–26A→G or c.14–28T→C), or GATA4 (c.14–26A→G or c.14–28T→C) under hypoxic conditions (Fig. 2E and Fig. S9B and S9C). Publicly available data[21-24] from ChIP sequencing (a technique designed to study genomewide protein–DNA interactions) of various human cell lines show the binding of GATA factors to regions within the first intron close to the mutated regions (Fig. S10D, S10E, and S10F). Both variants (c.14–26A→G and c.14–28T→C) also led to a significant increase in HIF-2α-mediated induction of *EPO* reporter gene expression (Fig. S9D), thus showing that these two variants affect the regulation of *EPO* transcription.

Finally, we investigated the effects of all three variants on endogenous *EPO* mRNA expression levels in patient-derived iPSCs. Given that HIF-2α and GATA factors have an established function in the regulation of hepatic EPO,[25,26] we differentiated iPSCs into hepatocyte-like cells (Fig. 2F). Our results showed a pronounced increase in *EPO* expression (by 3.4 to 15.5 times) in all mutant cells under hypoxic conditions ($P \leq 0.001$) (Fig. 2G), which was consistent with our RNA sequencing results (Fig. S8G and S8H) and showed an effect of the variants on *EPO* transcription. Collectively, these results showed that the identified variants in the *EPO* locus induced the upregulation of *EPO* expression. Together with the genetic data, segregation studies, and functional analysis, these variants can therefore be classified as pathogenic (class 4 or 5) and considered to be causal in the occurrence of erythrocytosis (see the ACMG criteria in Table S1).

ANALYSES OF PATIENTS' CIRCULATING EPO

Given the complexity of the involved mechanisms (variants associated with elevated *EPO* mRNA expression in patients' derived cells, with circulating EPO levels within the normal range) (Table 1), a full biochemical characterization of EPO was performed in collaboration with the French Anti-Doping Laboratory, which developed a high-end method to characterize EPO by isoelectric focusing in a pH gradient (pH of 2 to 6, separated into three areas denoted as "basic," "neutral," or "acidic" for simplicity[20]).

Samples that had been obtained from the six families described here were examined, in addition to plasma samples that had been obtained from patients with erythrocytosis associated with two *EPO* variants previously described (c.–136G→A and c.32delG).[14,15] In healthy persons, the isoelectric-focusing profile of circulating EPO is characterized by a distribution of up to 16 isoforms resulting from different glycosylated forms. Some variation was observed, but the main isoforms were typically distributed within the neutral or slightly basic pH range of the gel (Fig. 3B and Figs. S11 and S12). All the patient samples showed a modified isoelectric-focusing profile, which resulted in a shift in the distribution of EPO isoforms with the main isoforms present in the basic pH range of the gel (Fig. 3B). Further analysis showed that the distinctive basic pH profile that was observed in these patients, as compared with wild-type EPO, was due to some glycosylation modifications, which suggested a lower sialic acid level (linked to negative acidic charges[27]) as well as other modifications (Fig. S13).

We then examined whether the same isoelectric-focusing profile could be observed in patients with erythrocytosis associated with variants in *VHL* and high circulating levels of EPO, which could potentially lead to glycosylation defects owing to EPO overproduction. Their EPO profile closely resembled that of healthy adults, a finding that suggested that EPO in patients with *VHL* variants is produced mainly in the kidneys (Fig. 3C). In addition, we analyzed other cases involving patients with acquired erythrocytosis associated with

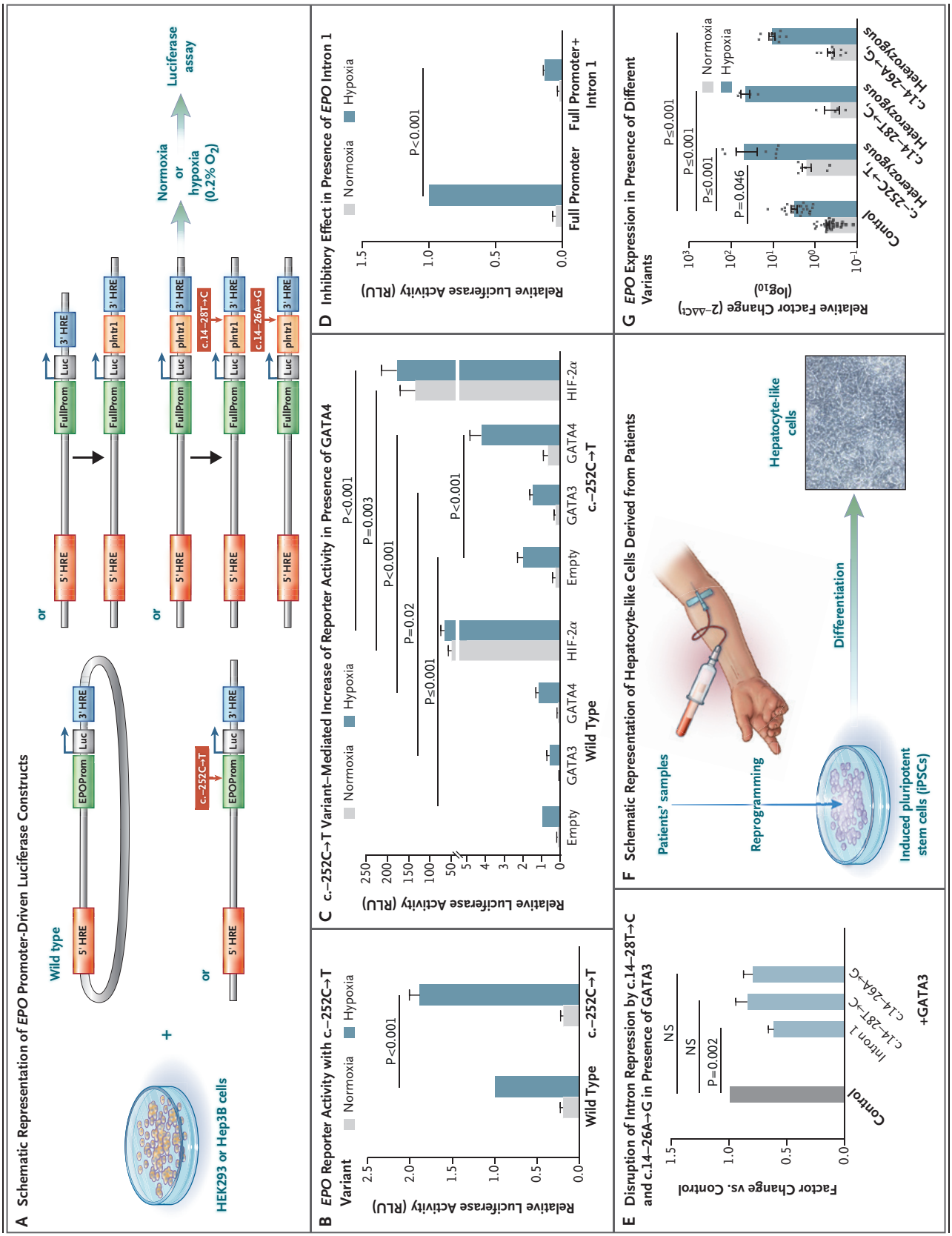


Figure 2 (facing page). Functional Studies of *EPO* Variants.

Panel A shows a schematic representation of the *EPO* promoter–driven luciferase (Luc) constructs, containing the distal kidney-inducible 5′-HRE (hypoxia response element) and liver-inducible 3′-HRE, that were used for functional assays of the c.–252C→T variant. Also shown is a schematic representation of the *EPO* proximal and distal regulatory element–driven luciferase constructs used for the functional assessment of a partial sequence of intron 1 (with the partial intronic sequence [pIntr1] containing the sequence from c.14–152 to c.15) and a schematic representation of the *EPO* regulatory element–driven luciferase constructs codriven by intron 1 used for the functional assessment of variants c.14–28T→C and c.14–26A→G. Human embryonic kidney (HEK293) cells were transiently transfected with an *EPO* promoter (EPO Prom) or enhancer constructs alone (Panel B) or in the presence of overexpressed HIF-2α, GATA3, and GATA4 (Panel C), exposed to normoxic and hypoxic conditions (0.2% oxygen) for 24 hours, and the effect of the c.–252C→T variant was assessed with the use of dual luciferase assays (Panel C). The effect of the addition of intronic fragment (Panel D), as well as the c.14–28T→C and c.14–26A→G variants (Panel E), in the presence of overexpressed GATA3 was assessed with the use of dual luciferase assays in HEK293 cells exposed to hypoxic conditions for 24 hours. Luciferase experiments showing overexpression controls with the use of immunoblotting are provided in Figure S17. Luciferase activity is reported as the induction as compared with wild-type control (Panels B and D) or empty expression vector (Panel C) under hypoxic conditions and represents the relative activity of two luciferases, firefly and Renilla, in relative light units (RLU). The factor change as compared with control (empty expression vector) was calculated (Panel E). Each bar represents the mean of four to eight different experiments performed in duplicate; I bars indicate the standard error. Panel F shows a schematic representation of the generation of hepatocyte-like cells derived from patients. Panel G shows quantification of *EPO* messenger RNA expressed in patients' induced pluripotent stem cells (iPSCs) differentiated into hepatocyte-like cells; the analysis was done by means of a TaqMan assay and is shown on a logarithmic scale. Two clones of each iPSC line were tested, and each point corresponds to a differentiation experiment. The term "ΔΔCt" refers to the relative quantification of gene expression, calculated on the basis of the difference in cycle threshold (CT) values between the target and housekeeping genes. The $2^{-\Delta\Delta Ct}$ values were calculated with the use of control group under normoxic conditions as the reference (equivalent to 1 [i.e., 10^0]). Induction factors of mean expression were 15.5, 14.7, and 3.4 as compared with control for the variants c.–252C→T, c.14–28T→C, and c.14–26A→G, respectively. In all panels, P values were calculated by means of a one-way analysis of variance or, alternatively, with a Kruskal–Wallis or Mann–Whitney test.

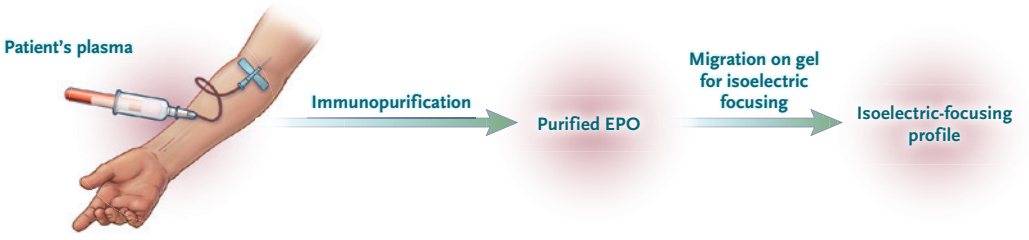
hepatic diseases.[28,29] These patients had a basic pH profile of EPO (Fig. 3C) that was very similar to the profiles observed in patients with the *EPO* variants identified in this study (Fig. 3B). Our results showed that the liver produces a more basic pH isoform of EPO, which is observed in the case of hepatopathies (Fig. 3C) and is associated with specific post-translational glycosylation modifications.

Given that in mammals EPO is produced by the liver during fetal life,[26] we next analyzed EPO samples obtained from preterm newborns. The isoelectric-focusing pattern seen in newborns showed the same basic pH profile as had been observed in our patients (Fig. 3D). Hence, our patients with erythrocytosis associated with *EPO* noncoding variants produced EPO that was qualitatively similar to EPO expressed by the fetal liver during development. By examining the EPO profile of a cohort of newborns from preterm to full term, we found that the EPO profile shifted as a function of developmental age, from a basic pH profile to a regular neutral pH profile that resembled the circulating EPO with an adult kidney–type glycosylation pattern. The liver-type glycosylation pattern was also identified in umbilical-cord blood samples obtained from neonates (all at ≥37 weeks' gestation), and the isoelectric-focusing profile was consistent with their developmental age (Fig. 3D).

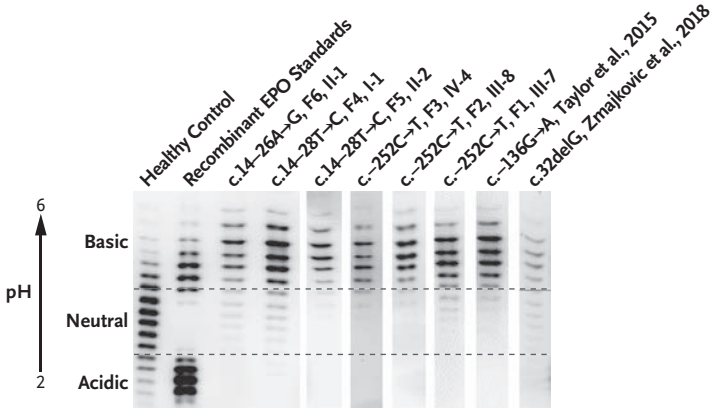
FUNCTIONAL STUDY OF EPO WITH LIVER-TYPE GLYCOSYLATION PATTERN

To study the properties of this particular EPO with a basic pH profile, we attempted to produce it in cell lines. However, we were unable to generate a correctly glycosylated EPO that resembled the normal circulating form in the blood and could serve as a control (data not shown). Activity assays of pure plasma that were performed on the human leukemia cell line UT-7/EPO, which expresses the EPO receptor and is EPO-dependent, were inconclusive (data not shown). Consequently, we purified and concentrated circulating EPO from blood donations obtained from healthy carriers, from phlebotomy samples obtained from patients with erythrocytosis carrying an *EPO* variant, and from cord blood in order to assess the activity of EPO with the basic pH profile. The activity of the purified EPO was measured by assaying the EPO–EPO receptor signaling pathway in UT-7/EPO cells, with quantification of STAT5

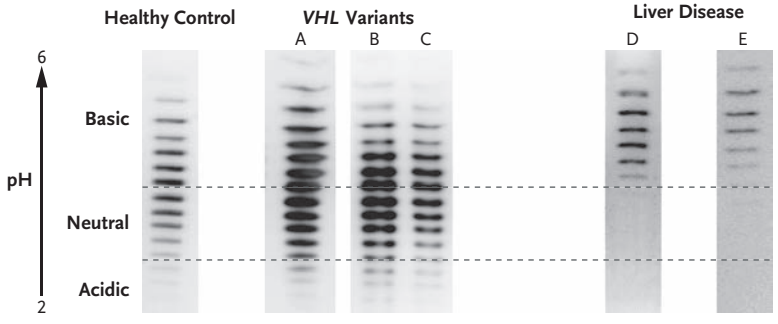
A Schematic Representation of EPO Assessment with Isoelectric-Focusing Profile



B Isoelectric-Focusing Profiles of EPO Isolated from Patients with Erythrocytosis Associated with *EPO* Variant



C Isoelectric-Focusing Profiles of EPO Isolated from Patients with Erythrocytosis Associated with *VHL* Variants with High EPO Level or Liver Disease



D Isoelectric-Focusing Profiles of EPO Isolated from Preterm Newborns

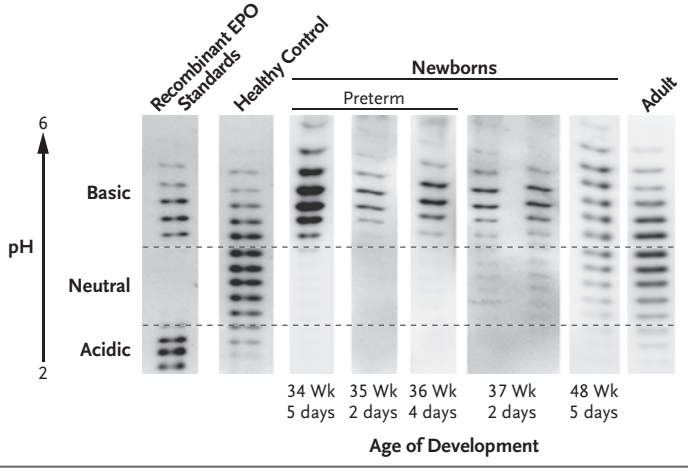


Figure 3 (facing page). Isoelectric Focusing of Circulating EPO.

Panel A shows a schematic representation of EPO assessment with the use of the isoelectric-focusing profile. Panel B shows isoelectric-focusing profiles of EPO isolated from patients with erythrocytosis associated with *EPO* variants, as compared with healthy control and recombinant EPO standard samples. Family (F) numbers and patient numbers are indicated. Results for two previously described variants are also shown (c.–136G→A and c.32delG).[14,15] Panel C shows results for three patients with erythrocytosis associated with *VHL* variants and a high EPO level (Patient A: 19,672 IU per liter; Patient B: 2195 IU per liter; and Patient C: 394 IU per liter) and for two patients with erythrocytosis associated with liver disease (Patient D with cirrhosis and splanchnic-vein thrombosis: 4 IU per liter; and Patient E with vascular cirrhosis: 48 IU per liter); the healthy-control profile is shown for comparison. Panel D shows isoelectric-focusing profiles of EPO isolated from preterm newborns (born at <37 weeks' gestation), as compared with recombinant EPO standard, healthy control, full-term newborn, and adult samples. The age of fetal development was calculated in weeks of the mother's amenorrhea.

phosphorylation with the use of AlphaScreen technology (Fig. 4A). We tested different EPO concentrations corresponding to the linear activity range of commercial EPO.

Figures S14B and S15A show isoelectric-focusing profiles of purified EPO and the dose–response curve. Our results indicated that EPO from patients consistently had higher activity than normal EPO at each of the tested doses, with significance ($P \leq 0.05$) observed at a level of 1.2 IU per milliliter (Fig. 4B). In addition, we purified EPO from cord blood of full-term newborns (≥37 weeks' gestation), whose samples predominantly expressed fetal liver EPO (Fig. 3D). Despite a mixed isoelectric-focusing profile, this EPO had significantly greater activity than the adult control EPO (Fig. 4C).

DISCUSSION

The regulation of *EPO* expression, a key hormone in erythropoiesis, has been studied for decades, but its complex regulatory mechanisms remain elusive. Our study not only elucidates cases of previously unexplained hereditary erythrocytosis but also provides mechanistic insights.

In this study, we identified three variants targeting crucial regulatory elements of *EPO*. In addition, we found that patients who had variants in *EPO* (including the c.–136G→A and c.32delG variants previously reported[14,15]) had fetal liver–like EPO with a modified glycosylation profile and associated enhanced activity. The concurrent discovery of an identical isoelectric-focusing profile in patients with liver diseases associated with erythrocytosis and in premature newborns (in whom fetal liver is the main source of EPO) strongly suggests that the liver is the site of production of "basic" EPO. This assumption is also supported by publications[30,31] showing differences in the glycosylation profiles of EPO produced by either the kidney or the liver. EPO from cord-blood serum was shown in previous studies to have a more basic pH profile than adult EPO,[30,31] a finding consistent with our observations. Although classification criteria clearly distinguished between the two types of EPO that are seen in controls as compared with patients (normal vs. basic pH distribution on isoelectric focusing), the presence of additional isoforms in the endogenous area for some patients could possibly reflect a higher residual expression of EPO from renal origin.

The modification of this profile related to glycosylation and the loss of negative (and acid) charges provides some clues for resolving the discrepancies between high mRNA level in cellular models and normal circulating EPO levels in patients. Nonsialylated glycoproteins have a much shorter in vivo half-life than sialylated ones because terminal galactose residues are exposed, recognized, and captured by asialoglycoprotein receptors in hepatocytes and the kidney.[32,33] In addition, the affinity of EPO for its receptor is inversely related to the sialylation of EPO carbohydrate.[34,35] Because the EPO receptor–mediated uptake is a major catabolism pathway, desialylated EPO is therefore less stable.[36] The glycosidase experiments that we performed did not completely elucidate the differences between wild-type and "basic" EPO owing to the complex structure of glycosylations of EPO, and changes in the sialic acid content constitute only a part of the explanation. Nevertheless, a lower degree of fully terminated glycosylation may lead to a lower stability of circulating EPO, which could explain the serum EPO level within the normal range in our patients with *EPO* variants, despite high EPO mRNA levels in the cellular model. The likely shorter half-life of the hepatic-like EPO may also explain the same discrepancy that

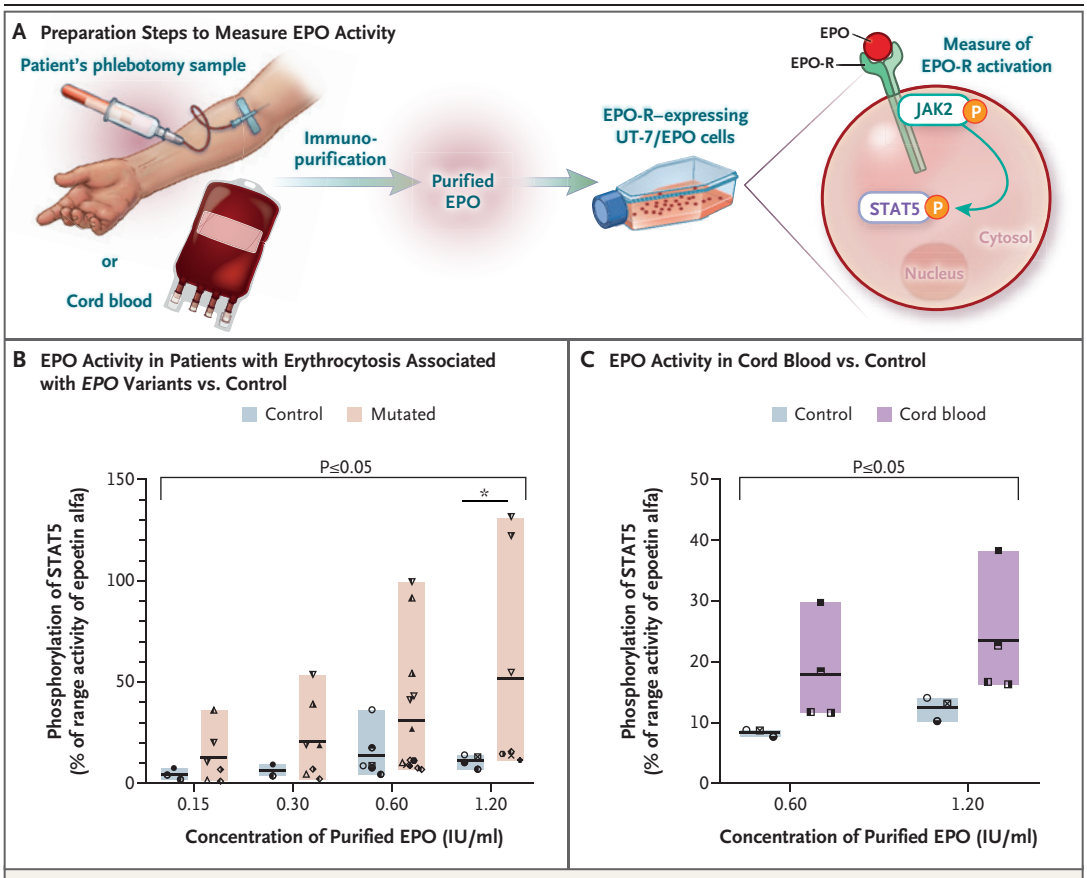


Figure 4. Activity of EPO Purified from Plasma of Patients and from Cord Blood.

Panel A shows a schematic representation of the preparation steps for measuring EPO activity. Phosphorylation of endogenous STAT5 in the human leukemia cell line UT-7/EPO, which expresses the EPO receptor (EPO-R) and is EPO-dependent, was stimulated by the different doses of purified EPO and was measured with the use of the AlphaLISA SureFire technique. Panel B shows the activity of EPO in purified samples obtained from patients with erythrocytosis associated with *EPO* variants, as compared with control. Panel C shows the activity of EPO in purified samples of cord blood, as compared with control. In both panels, results are presented as percentages related to the activity of the recombinant EPO epoetin alfa (100% corresponding to the maximal activity at high concentration). In the box plots in Panels B and C, the center line represents the mean, and the top and bottom of the box the range. The identity of each sample is indicated in Figure S15B and S15C, according to the small symbols on the box plots. P values were calculated by means of two-way analysis of variance. Per-concentration significance, as assessed by a Tukey post hoc test, is indicated by the asterisk.

has been described with the c.32delG *EPO* variant studied in Hep3B cells,[15] as well as in studies of *EPO* expression during the development of rat models.[37]

In addition, differences in glycans could be observed for other proteins, such as γ-glutamyltransferase, depending on the site of production (kidney or liver).[38] A similar mechanism may be taking place in the case of EPO, and thus, a different site of production from the renal interstitial cells, probably a liver-specific site, is implicated.

We established that this modified EPO, isolated from patients and cord blood, showed greater activity than adult renal EPO. Moreover, our findings may explain why, although renal cells begin to express EPO at birth, the decrease in expression of a more-active liver EPO leads to a decrease in the red-cell count in newborns, usually 4 to 6 weeks after birth.[39] In patients with *EPO* variants, only hepatic-like EPO is observed, which suggests a weak production of the EPO by the kidney. The same phenomenon is observed in the presence of EPO drugs (exogenous form), which have been shown to have higher activity than the wild-type EPO, leading to a reduction in endogenous EPO production by the kidneys.[40]

Regarding the molecular mechanisms responsible for the silencing of the liver expression of EPO after birth, our study allowed us to identify novel regulatory regions involved in GATA transcription factor–dependent regulation of *EPO* expression. Using reporter gene assays, we found that intron 1 possesses a negative regulatory region. Although the fact that GATA factors regulate *EPO* expression is well established,[26,41] their precise role in tissue-specific and complex interplay with proximal and more-distal regulatory regions remains to be ascertained. New therapeutic targets may be identified that can reactivate *EPO* expression by the liver in patients with acute renal failure.

Finally, our results resolved some cases of hereditary erythrocytosis that have been diagnosed in patients with gain-of-function variants in *EPO*. Erythrocytosis in these patients has been characterized by fetal liver–like EPO expression with modified glycosylation and enhanced activity. All the *EPO* variants that were identified in our patients were located in noncoding regions. Given the complexity of the *EPO* regulatory region,[3,17,42-46] the expansion of *EPO* screening in diagnostic DNA sequencing is recommended for unresolved cases of hereditary and idiopathic erythrocytosis. In addition, our results showed that simple EPO quantification is no longer sufficient for the diagnosis of erythrocytosis; qualitative analyses of the glycosylation pattern, such as isoelectric focusing, will be useful in diagnostic screening protocols. On a broader scale, isoelectric focusing could serve as a potent tool to elucidate more-common cases of acquired idiopathic erythrocytosis, especially those associated with hepatic pathologic conditions. Ongoing research in this field may unravel additional facets of the regulation of EPO and pave the way for new therapeutic applications.

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