EPO Receptor Gain-of-Function Causes Hereditary Polycythemia, Alters CD34+ Cell Differentiation and Increases Circulating Endothelial Precursors

Silverio Perrotta1, Valeria Cucciolla2, Marcella Ferraro1, Luisa Ronzoni3, Annunziata Tramontano2, Francesca Rossi1, Anna Chiara Scudieri2, Adriana Borriello2, Domenico Roberti1, Bruno Nobili1, Maria Domenica Cappellini3, Adriana Oliva1, Giovanni Amendola4, Anna Rita Migliaccio5, Patrizia Mancuso6, Ines Martin-Padura6, Francesco Bertolini6, Donghoon Yoon7, Josef T. Prchal7, Fulvio Della Ragione2*

1 Department of Pediatrics, Second University of Naples, Naples, Italy, 2 Department of Biochemistry and Biophysics, “F. Cedrangelo”, Second University of Naples, Naples, Italy, 3 Foundation Ospedale Maggiore Policlinico IRCCS, University of Milan, Milan, Italy, 4 Ematologia-Oncologia Pediatrica, Ospedale di Nocera Inferiore, Nocera Inferiore, Italy, 5 Mount Sinai School of Medicine, New York, New York, United States of America, 6 Laboratory of Hematology-Oncology, European Institute of Oncology, Milan, Italy, 7 Hematology Division, School of Medicine, University of Utah and VAH, Salt Lake City, Utah, United States of America

Abstract

Background: Gain-of-function of erythropoietin receptor (EPOR) mutations represent the major cause of primary hereditary polycythemia. EPOR is also found in non-erythroid tissues, although its physiological role is still undefined.

Methodology/Principal Findings: We describe a family with polycythemia due to a heterozygous mutation of the EPOR gene that causes a G→T change at nucleotide 1251 of exon 8. The novel EPOR G1251T mutation results in the replacement of a glutamate residue by a stop codon at amino acid 393. Differently from polycythemia vera, EPOR G1251T CD34+ cells proliferate and differentiate towards the erythroid phenotype in the presence of minimal amounts of EPO. Moreover, the affected individuals show a 20-fold increase of circulating endothelial precursors. The analysis of erythroid precursor membranes demonstrates a heretofore undescribed accumulation of the truncated EPOR, probably due to the absence of residues involved in the EPO-dependent receptor internalization and degradation. Mutated receptor expression in EPOR-negative cells results in EPOR and Stat5 phosphorylation. Moreover, patient erythroid precursors present an increased activation of EPOR and its effectors, including Stat5 and Erk1/2 pathway.

Conclusions/Significance: Our data provide an unanticipated mechanism for autosomal dominant inherited polycythemia due to a heterozygous EPOR mutation and suggest a regulatory role of EPO/EPOR pathway in human circulating endothelial precursors' homeostasis.

Introduction

Erythropoietin (EPO) is a key cytokine, produced mainly in peritubular renal cells but also in the liver, that modulates the growth, survival, and differentiation of erythroid progenitor cells, leading to tight control of red blood cell production. Its receptor (EPOR) is a homodimeric transmembrane protein of 508 amino acids in humans (507 in mice) that belongs to a superfamily of cytokine receptors, which includes receptors for GM-CSF and interleukin-3 and -6 [1]. As with other members, the extracellular ligand binding region of EPOR contains four conserved cysteine residues and a WSXWS motif.

The human EPOR gene is located on chromosome 19 and contains eight exons [2 and references therein.] The first five exons encode the extracellular region that embraces the high EPO affinity of A, B, D helix site-1 and the low Epo affinity of A, C helix site-2 interactions, with 7 beta-strand bipartite binding sites in appositioned EPOR dimmers. Exon 6 encodes the membrane spanning domain, while the two intracellular receptor domains are encoded by exons 7 and 8 [2 and references therein].

The receptor does not possess any kinase activity, but its intracellular region binds to JAK2 tyrosine kinase that is essential for EPO signaling. EPOR engagement stimulates JAK2 phosphorylation at Y1007/1008 residues [3]. In turn, activated JAK2 (in concert with other kinases) phosphorylates eight conserved tyrosines in cytoplasmic domain [4]. These phosphotyrosine (PY) sites of EPOR function as docking sites for the binding of molecules containing SH2/SH3 motifs to EPOR. One subset of PY site–recruited factors (PY 402, 430, 432) coordinates negative feedback of EPO signaling. EPO’s positive signals are determined
Results and Discussion

The propositus (P1, Figure 1A) was referred from a group of 114 patients included in the Italian registry of congenital erythrocytosis/polycythemia. The index patient is a 14-year-old child who, at 7 years of age, was evaluated for persistent headaches and leg muscle cramps. He had an elevated hemoglobin level and hematocrit (21 g% and 66%, respectively), no splenomegaly, and normal blood pressure. White blood cell and platelet counts were normal. The patient’s 41-year-old father (P3) presented at 19 years of age with elevated values for hemoglobin and hematocrit (20.1 g% and 64%, respectively) and normal white-cell and platelet counts. He suffered from persistent headaches and had a mild mitral valve insufficiency and a liver steatosis. Both P1 and P3 were regularly phlebotomized with symptomatic relief. The propositus’ paternal grandfather was also reported as “polycythemic”. He died at 55 years of age of liver cirrhosis. The hemoglobin level and hematocrit were also elevated in the paternal uncle (P4) and his daughter (P5), but these patients have scarce clinical symptoms and, thus, were not phlebotomized. The serum EPO was <3 mU/mL (normal range, 4 to 32 mU/mL) in all the four polycythemic family members, while hemoglobin oxygen affinities (p50) were normal.

All patients were screened for defects in the Von Hippel-Lindau, HIF-1alpha, HIF-2alpha, PHD1-3, JAK2, and EPOR genes. No mutations were found in the Von Hippel-Lindau, HIF-1alpha, HIF-2alpha, PHD1, PHD2, PHD3, and JAK2 genes. However, a heterozygous G→T change at nucleotide 1251 in exon 8 of the EPOR gene was detected in the index patient (Figure 1A). This novel mutation (EPOR G1251T) caused the replacement of a glutamic acid residue with a stop codon at amino acid 417, which corresponds to residue 393 of the mature receptor. The EPOR G1251T mutation segregated with the polycythemic status, as demonstrated by the EPOR analysis of other family members (Figure 1B). This genetic change resulted in the synthesis of a truncated receptor lacking the cytosolic tyrosines subjected to phosphorylation, except Y344.

Since our patients had a very low serum EPO level, we investigated the growth and differentiation of erythroid precursors in the absence of exogenously added EPO with normal and polycythemia vera (PV) cells used as controls. PV cells were chosen as their erythroid precursors are EPO hypersensitive.

In a first set of experiments, the proliferation of erythroid precursors was investigated by employing liquid cultures of nucleated cells (PV) and untreated normal control mononuclear cells before and after stimulation with recombinant EPO. Significant growth rate differences were observed between EPOR G1251T and PV patients that expressed the erythroid receptor. The EPOR G1251T precursors grew 2–3 times faster than cells from PV. Thus, the cells from EPOR G1251T patients increased about 16-fold after 12 days compared to a 6-fold increase of the PV subjects (Figure 2A). Under the same condition, normal erythroid precursors underwent apoptosis. Significant growth rate differences between EPOR G1251T and PV patients were evident at all examined time-periods (Figure 2A).

Glycophorin A expressing cells were analyzed after 14 days of proliferation without exogenously added EPO (i.e. at 0.4 mU/mL EPO concentration) as reported in Figure 2B. The percentages of EPOR G1251T cells and PV patients that expressed the erythroid marker were 53±5% and 13±2%, respectively.

When, the same experiment was performed in the presence of 3 U/mL EPO, no significant difference of erythroid maturation was observed (Figure 2B).
A second set of experiments was performed employing CD34+ cells prepared from EPOR G1251T and PV patients to highlight phenotypic differences of the early hematopoietic precursors from these two forms of erythrocytoses. As shown in Figures 2C and 2D, while a significant percentage (51.5±4.1%) of peripheral CD34+ cells from P1 (grown for 14 days without exogenously added EPO) expressed glycophorin A, the antigen was not detectable in peripheral CD34+ cells from PV patients cultured under the same conditions.

When CD34+ cells were cultured on soft agar with minimal EPO (0.4 mU/mL) marked differences in the number and size of colonies were observed after 14 days. As shown in Figure 2E, CD34+ cells of P1 formed large visibly hemoglobinized colonies while only few small, pale, poorly hemoglobinized colonies were seen in CD34+ cells cultures from PV patients (Figure 2E). Identical findings were obtained with CD34+ cells from P3, P4 and P5 patients (data not reported). We also prepared the DNA from the largest colonies of P1 and examined the status of the EPOR gene. No gene conversion was observed and the heterozygosity of EPOR G1251T was maintained.

Finally, no difference in the number and size of colonies was observed after 14 days when CD34+ cells from control subjects, PV patients or PFCP patients were cultured on soft agar in the presence of 3 U/mL EPO (data not reported).

It has been suggested, although still debated, that EPO/EPOR pathway plays a role in the endothelial cell homeostasis. Thus, we analyzed the number of circulating endothelial precursors (CEPs) of all PCFP patients by flow cytometry. CEPs were identified as CD45dim/CD34+/VEGFR2+ peripheral cells [32]. Figure 3A depicts an example of the flow cytometry analysis performed. CEPs were 710±5 cells/mL in the EPOR-mutated subjects (Figure 3B), which is about 20-fold higher than the CEPs in the age-matched controls. The CEP level of 3 examined PV patients, all having a classical JAK2 mutation (JAK2V617F), were similar to the CEPs content of EPOR G1251T subjects (Figure 3B).
Figure 2. Phenotypical features of erythroid precursors and CD34+ cells from the affected patients. Panel A. Growth curve of blood erythroid precursors from the four EPOR G1251T subjects, three subjects affected by PV JAK2 V617F homozygotes, and three healthy subjects. Erythroid precursors were grown from peripheral mononuclear cells as described (Migliaccio et al., 2002) without adding recombinant EPO. The estimated concentration of EPO in the growth medium was about 0.4 mU/mL. Data represent mean ± SEM (n = 3 per cell type), and are representative of 3 experiments. Panel B. The graphic reports the percentage of BFU-E cells (evaluated by glycophorin A expression after 14 days growth) in liquid cultures of erythroid precursors from the P1 patient and two PV patients (JAK2 V617F homozygotes). The cells were cultured in the absence of exogenously added EPO. Data represent mean ± SEM (n = 3 per cell type), and are representative of 2 experiments. Panel C. Fluorescent activated cell scanner (FACS) analysis of liquid cultures of peripheral purified CD34+ cells grown for 14 days in with minimal EPO. The erythroid precursors were prepared from the P1 subject and from a patient affected by PV associated with a classical JAK2 mutation (JAK2 V617F homozygote). GlyA+ means glycophorin A positive cells. The results are representative of three independent experiments that gave superimposable results. Panel D. FACS analysis of samples shown in panel C. The panel reports the analysis of GlyA levels in cells plotted versus forward scatter. Panel E. Peripheral CD34+ cells were growth on soft agar in the absence of EPO. The images report the features of colonies from the P1 subject (b, d) or from a subject affected by PV JAK2 V617F homozygote) (a, c). The results are representative of four independent experiments that gave superimposable results. doi:10.1371/journal.pone.0012015.g002
Primary Polycythemia and EPOR

A

B

C

D

E

C, Healthy subjects
FP, Familial polycythemia (EpoR mutated)
PV, Polycythemia vera

EPOR transcript relative expression

Erythroid cells
CEPS
The growth characteristics of the EPOR G1251T CD34+ cells and the increased levels of peripheral CEPs prompted us to investigate EPOR mRNA and protein levels during CD34+ erythroid differentiation in vitro.

The expression of both EPOR mRNA alleles in erythroid precursors from the four patients was evaluated by semiquantitative PCR (Figure 3C) and real-time PCR (Figure 3D) after 7 days of cultures in the presence of EPO. Both methods demonstrated that mutant EPOR G1251T and wild type EPOR alleles were expressed at similar levels and that the total EPOR transcript of the EPOR G1251T erythroid precursors corresponds to the EPOR mRNA of erythroid precursors of an healthy subject (Figure 3D). This is in accord with results of a previous study on PCFC, although different PCR methods were employed [19].

The real-time PCR approach was also employed to evaluate the expression of the two EPOR alleles of primary cultures of CEPs (P1, P3 and P5 patients). The results obtained are identical to those obtained on the erythroid precursors in that both mutant EPOR G1251T and wild type EPOR alleles were expressed at similar levels (data not shown). It is to underline that CEPs EPOR mRNA is easily detectable and that it corresponds to about 10% of EPOR mRNA of erythroid precursors, the cells that probably express the major amount of this transcript in the human body (Figure 3E).

In conclusion, the evaluation of levels of the two EPOR mRNA forms indirectly argues that similar amounts of normal and truncated protein receptors may exist in EPOR-expressing cells as reported in previous studies on PCFC.

In order to verify this conclusion, the status of the EPOR protein on the membrane of erythroid precursor cells was investigated. The lack of specific antibodies against the N-terminal region of EPOR has so far hampered direct biochemical analyses of the EPOR peptide. Recently, we have identified and characterized in detail a highly specific antisera against the extracellular domain of the receptor [33]. To rule out nonspecific crossreactivity by the EPOR antibody and identify the molecular weight of the truncated receptor, we prepared the recombinant wild type and mutated EPOR proteins with an in vitro transcription and translation (IVT) kit. Moreover, we expressed the native and truncated EPOR in a human EPOR-negative cell line, i.e. K562 cells, for further positive controls. As shown in Figure 4A, the antisera against the extracellular domain recognized the wild type EPOR form at 66–67 kDa and the mutated EPOR G1251T form at about 52–53 kDa.

When the isolated cell membrane fractions from the patient were analyzed, we observed the presence of a strong band at the mutated form and a very faint band at the wild type EPOR. The finding was observed in either CD34+ cells grown for 7 and 10 days in the presence of 3 U/mL EPO (Figure 4B) or in cells cultured for 7 days in minimal EPO level (0.4 mU/mL) (Figure 4C). The analysis of membrane fractions from control erythroid precursors showed a barely detectable band of the wild type protein.

It is to underline that when CD34+ cells (both from controls or PCFC subjects) were growth in the presence of EPO they showed similar differentiation as demonstrated by their glycoprotein A content (Figure 4B). Thus, changes in the truncated EPOR protein levels cannot be ascribed to differences in cellular populations due to variable degree of differentiation.

The unanticipated observation that, even in the presence of high EPO, the lack of a large part of the receptor cytoplasmic domain results in the cell membrane accumulation of mutated EPOR, suggested that the truncated receptor has a slower membrane internalization/degradation and an increased half-life due to the absence of sequences required for receptor internalization and degradation.

In order to confirm this hypothesis, we incubated CD34+ cells with a proteasome inhibitor, i.e. LLnL, that has been previously reported to induce membrane EPOR accumulation by hampering the receptor removal [34]. Thus, CD34+ cells were cultured for 7 days with EPO and, then, treated for 4 hours with the proteasome inhibitor. As shown in Fig. 4D, LLnL treatment causes the increase of wild-type EPOR while scarce change of mutated EPOR level was observed. The finding suggests that the turn-over time of the two forms was considerably different, being the mutated form remarkably more stable.

The observed difference of receptor turn-over is also in accord with data obtained in UT-7 cells, showing EPOR internalization and removal requires ubiquitination of the receptor’s C-terminal region [34]. In particular, members of the beta-Trcp family have been shown to participate in the E3 ligase activity responsible for EPOR ubiquitination [35]. Beta-Trcp binds to Ser 462 within the intracellular part of the receptor and contributes to EPOR ubiquitination in the presence of the hormone. The ubiquitination mediated by beta-Trcp is a crucial signal for the C-terminus degradation of EPOR because the point mutation of Ser 462 to Ala blocks EPOR targeting to the proteasome, thereby leading to sustained activation of EPOR[S462A]. It is interesting that BaF3 cells, expressing a mutated EPOR unable to bind beta-Trcp, are hypersensitive to EPO, suggesting beta-Trcp-mediated ubiquitination represents a negative modulator of EPO-induced cellular
Figure 4. Analyses of EPOR protein in erythroid precursors. Panel A. Western blot analysis of cellular membranes from the following samples (from the left to right): i) UT-7 cells; ii) untransfected K562 cells (Con); iii) wild-type EPOR transfected K562 cells (WT-EPOR); iv) mutated EPOR transfected K562 cells (MutEPOR); v) in vitro transcription/translation (IVTT) control mixture; vi) in vitro transcribed and translated wild-type EPOR, and vii) in vitro transcribed and translated mutated EPOR. K562 cells were transfected employing pMT21 plasmids encoding wild-type or mutated EPOR, while pcDNA3.1 plasmids were employed in the IVTT experiments. UT-7 cells were employed since these cells contain abundant amounts of wild-type EPOR (Della Ragione et al., 2007). Immunoblotting was performed with the antiserum against the N-end of EPOR. The bottom image is the filter, before immunoblotting, and colored with Red Ponceau. The image confirms equal loading of membrane proteins (lanes 2, 3 and 4) and IVTT assay mixtures (lanes 6, 7 and 8). The immunoblotting is representative of 4 experiments. Panel B. The image on the left reports the immunoblotting analysis of membranes from the following samples (from left to right): i) peripheral CD34+ cells from a healthy subject (Con) grown for 7 days with EPO (3 U/mL); ii) peripheral CD34+ cells from patient P1 (MutEPOR) growth for 7 days with EPO (3 U/mL); iii) peripheral CD34+ cells from a healthy subject (Con) grown for 10 days with EPO (3 U/mL); and iv) peripheral CD34+ cells from patient P1 (MutEPOR) grown for 14 days with EPO. The immunoblotting on the right reports cell membranes from K562 cells cotransfected with the wild-type and mutated EPOR pMT21 plasmids as standard for the two EPOR forms. The immunoblotting was performed with the antiserum against the N-end of EpoR. The filter was also re-analyzed by antibodies against glucophorin A (immunoblotting at the center). The bottom image was taken to the filter colored with Red Ponceau before immunoblotting. The image confirms equal loading of membrane proteins. The image is of 3 experiments. Panel C. The image on the left reports the immunoblotting analysis of membranes from the following samples (from left to right): i) peripheral CD34+ cells from a healthy subject (Con) cultured for 7 days without minimal EPO (0.4 mU/mL); ii) peripheral CD34+ cells from patient P1 (MutEPOR) cultured for 7 days without EPO. The immunoblotting at the right reports cell membranes from K562 cells cotransfected with both the wild-type and mutated EPOR pMT21 plasmids. The immunoblotting was performed using the antiserum against the N-end of EPOR. The bottom image was taken to the filter colored with Red Ponceau.
proliferation [35]. This finding is similar to our observation of a strong EPO hypersensitivity of the erythroid precursors possessing the truncated receptor. In addition, the mechanism is in accord with a recent investigation demonstrating that phosphorylated Y129, Y431, and Y479 in the EPOR cytoplasmic domain bind the p85 subunit of PI3 kinase on EPO stimulation and individually are involved in mediating EPO-dependent EPOR internalization [36].

In order to highlight the biochemical consequences of the truncated receptor accumulation, the possibility that the two EPOR isoforms might interact to form a heterodimer was investigated. Thus, the truncated and wild-type EPORs were overexpressed in K562 cells and membrane proteins were incubated with an antiserum directed against the C-terminus. The immunoprecipitated materials were then analyzed with the antiserum directed against the N-terminus and the occurrence of both the native and the truncated proteins was shown. The result obtained demonstrated that the two EPOR isoforms might undergo heterodimerization (Figure 4E). However, although the input material contained equal amounts of the two EPOR isoforms and almost all the wild-type receptor was immunoprecipitated, a large excess of the truncated receptor remained in the supernatant (Figure 4E). This argues that the wild-type receptor, and thus the truncated EPOR, preferentially form homodimers.

Therefore, we evaluated whether high levels of homodimers of truncated EPOR might result in excessive EPO signaling and/or in an EPOR autoactivation event. To validate these hypotheses, the truncated EPOR was overexpressed in K562 cells and the effect on receptor autophosphorylation and Stat5 phosphorylation was evaluated. As shown in Figure 5A, transfection of the truncated receptor induced a number of biochemical changes (i.e., receptor autophosphorylation and STAT5 phosphorylation), even in the absence of exogenously added EPO, suggesting the possibility of autoactivation and/or hypersensitivity of receptor. Since these events occur at level of truncated EPOR similar to those observed in erythroid precursors cells, it is likely that these receptor features might be observed in these cells.

Then, we investigated the activity of the EPOR-JAK2-STAT5 pathway in CD34+ from control and EPOR truncated subjects, grown for 7 days with or without exogenously added EPO. As shown in Figures 5B and 5C, in the presence of 3 U/mL EPO, we found a clear increase of receptor and STAT5 phosphorylation. Although we were unable to directly demonstrate the phosphorylation of the truncated EPOR in the absence of the cytokine, we observed, under this condition, the activation of EPOR-dependent pathway by detecting STAT5 phosphorylation in CD34+ cells cultured without EPO (Figure 5D). The finding was also confirmed by the activation of Erk1/2, which was significantly increased in cells from EPOR truncated subjects, grown for 7 days with EPO (Figure 6A). In the presence of minimal amounts EPO, we also found increased Erk1/2 phosphorylation, which suggests greatly augmented activity of the receptor (Figure 6B).

An increase of p27Kip1 (an inhibitor of cell cycle progression) has been reported during erythroid differentiation of experimental models of erythropoiesis [37]. As reported in Figure 6C, we also observed an increase of p27Kip1 on different days of CD34+ growth in the presence of EPO. Intriguingly, in cells grown for 7 days with EPO, we found an increase of p27Kip1 in cells from the EPOR G1251T patient compared with cells of the control subject (Figure 6D).

In conclusion, in this study, we report that the gain-of-function EPOR G1251T mutation results in a marked alteration of growth and differentiation of human CD34+ and in an increase of erythroid progenitors’ surface EPOR peptide. Moreover, we observed a profound increase of circulating endothelial precursors in the affected subject. Our data elucidate how the heterozygous EPOR gene mutation causes a dominantly inherited polycythemia phenotype and erythroid progenitors’ EPO hypersensitivity. We demonstrate for the first time that, although both alleles of the patients were transcribed at similar rates in erythroid precursors and roughly the same as the normal alleles of healthy subjects, the truncated receptor accumulates at very high levels on cellular membranes. This leads to augmented activation of growth and erythroid differentiation of CD34+ observable at EPO levels lower that that necessary to activate CD34+ of PV patients. EPO hypersensitivity might be due to both: i) the remarkable accumulation of truncated EPOR and its autoactivation, and ii) loss of the negative regulatory domain of the EPOR G1251T mutant.

The CD34+ cells proliferation and differentiation, observed without exogenous EPO, also demonstrated the EPOR sequence, including Y344, is sufficient to activate the proliferation and differentiation of human erythroid precursor cells. In the same context, the truncated EPOR-dependent activation of Erk2 may not require phosphorylated Erk2 may not require PY480 or phospholipase C-gamma for its activation, as reported by others [2].

The importance of the EPO-EPOR system in erythropoiesis and vasculogenesis was established using mutant mice lacking either the Epo or EpoR gene [38]. Although these observations are consistent with participation of EPOR in vasculogenesis, the precise contribution of EPOR function in nonhematopoietic tissue remains to be defined, and is the object of intense debate. It has been described that EPO administration can increase the number of CEPs in healthy subjects. It has been postulated that this increase might be due to an EPO-derived stimulation of cytokine production. Our observation that the EPOR G1251T patients with activated EPO-EPOR signaling have an increased number of CEPs suggests the CEPs increase may be due to a constitutive EPO receptor activation in this population. An increase in circulating CD34+ cells has been described in patients carrying...
JAK2 mutations and suffering from myeloproliferative diseases [39]; to our knowledge, this is the first report demonstrating an increased number of endothelial precursors in the circulation of patients with gain-of-function EPOR mutations. It remains to be established whether this is due to increased CEP production, increased CEP mobilization, or both mechanisms.

**Materials and Methods**

**Mutational analysis and evaluation of EPOR mRNA isoforms**

Blood samples were obtained from the index patient, his parents and relatives, from healthy controls, and from four patients with
PV (with homozygous JAK2V617F mutation). All patients gave written informed consent on entering the study, which was approved by the research ethics committee of Second University of Naples, Italy, and the study was conducted according to the Declaration of Helsinki. DNA and RNA were prepared by standard methods. To screen for mutations of the EPOR gene, exons 7 and 8 with exon-intron boundaries were amplified by PCR using the following method and primers:

1. PCR was performed with 30 cycles of denaturation (1 min, 94°C), annealing (1 min, 60°C), and elongation (1 min, 72°C) and the amplified products were analyzed on 2% agarose gels, stained with ethidium bromide. Oligonucleotides used to amplify either exon 7 (362 bp) or 8.1 (301 bp) and exon 8.2 (333 bp) were as follows:
   - Exon 7 forward: 5'-GCCTCTATGACTGGGAGTGG-3'
   - Exon 7 reverse: 5'-GCGCTCTGAGAGGACTTCC-3'
   - Exon 8.1 forward: 5'-GCCTGGGCTTCCCTGCTTCTTGC-3'
   - Exon 8.1 reverse: 5'-TTCGAGGCCAAAGCAGATGAGCA-3'
   - Exon 8.2 forward: 5'-TATCTGGTGCTGGACAAATGGTT-3'
   - Exon 8.2 reverse: 5'-CTGCAGCCTGGTGTCCTAAGAGC-3'

2. The amplified products were purified and sequenced.

Two independent methods were developed for quantifying the expression of the EPOR alleles (i.e., wild-type and mutated forms). First, we designed a primer incorporating a mismatched base, corresponding to the allele with the G1251T mutation, to engineer a restriction site for NdeI in the PCR product. After the PCR reaction, the assay mixture was digested with the NdeI enzyme (Invitrogen, Carlsbad, CA, USA) and wild type and mutated EPOR plasmids. Further details on the methods, as well as on the primers employed, will be provided on request.

Erythroid precursor cultures

Liquid cultures of erythroid precursors from peripheral blood were prepared by two methodologies. The first utilized peripheral blood mononuclear cells as a source of erythroid progenitors [31], while the second employed the peripheral blood CD34+ cells as a source of progenitors [40]. Soft agar colony assays were performed as described [41]. The colonies were scored after 0, 7, and 14 days and their images captured after 14 days.

Circulating endothelial cell evaluation

Circulating endothelial precursors were measured by six-color flow cytometry as previously described [32]. Primary cultures of circulating endothelial precursors were prepared as previously described [32].
Plasmids, cell lines, and protein analysis

The wild-type coding sequence of human EPOR, cloned into the pMT21 expression vector, was kindly provided by Dr. A. D’Andrea. The sequence was mutagenized at the 1251 (G→T) position, and the wild-type and mutated EPOR sequences were subcloned into the pcDNA3.1 plasmid. These pcDNA3.1 vectors were used to prepare in vitro the respective proteins by TNT kits (Promega Italia, Milan, Italy). pMT21 plasmids containing the wild-type and mutated EPOR coding sequences were transiently transfected in the human K562 cell line (a negative erythropoietic EPOR cell line) by standard procedures [33,42]. The treatments with N-Ac-Leu-Leu-norLeucinal (LLnL) (Merck Biochemicals, Darmstadt, Germany) were performed as reported in [34]. Cell membranes were prepared with the Qproteome Cell Compartment Kit (Qiagen, Valencia, CA, USA), while cytosol and nuclei were obtained using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL) [33]. Immunoblotting procedures and immunoprecipitation experiments were essentially performed as described [33,42].

The following antibodies were employed in the immunoblotting and immunoprecipitation experiments. Goat polyclonal antisera directed against the extracellular domain of EPOR from Abscam (Abcam, Cambridge, MA); rabbit polyclonal anti C-end of EPOR, Stat5, and phospho-Erk1/2 and mouse monoclonal antibodies anti Erk1/2 and anti-phosphoryrosine (PY20) were from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal antibodies anti phospho-Stat5 were from Upstate Biotechnology (Upstate Biotechnology, Charlotteville, VA); monoclonal antibodies against p27kip1 were from Transduction Laboratories (Transduction Laboratories Lexington, KY).

Acknowledgments

We sincerely thank Dr. Carmela Migliaccio for mutational analysis of the EPOR gene and Dr. Luciana De Vito for preliminary immunoblotting. We wish to thank the family for their cooperation.

Author Contributions

Conceived and designed the experiments: SP DR MDC AO FB JTP FDR. Performed the experiments: VC MF LR AT FR ACS AB DR ARM PM IMP. Analyzed the data: SP VC MF LR AB BN MDC ARM FDR. Contributed reagents/materials/analysis tools: GA. Wrote the paper: SP.

References

1. Barber DL, D’Andrea AD (1992) The erythropoietin receptor and the molecular basis of polycythemia. Semin Hematol 29: 295–304.
2. Huang LJ, Shen Y-M, Buht GB (2010) Advances in understanding the pathogenesis of primary familial and congenital polycythemia. Brit J Haematol 148: 844–852.
3. Feng J, Winshun BA, Matsuda T, Kohudhe F, Kerr IM, et al. (1997) Activation of Jak2 catalytic activity requires phosphorylation of Y1007 in the kinase activation loop. Mol Cell Biol 17: 2497–2501.
4. Longmore GD (2006) A unique role for Stat5 in recovery from acute anemia. J Clin Invest 116: 626–629.
5. Pellestor S, Girgis S, Furushoki-Tao M, Howell S, Bile JN (2006) Two domains in the erythropoietin receptor are sufficient for Jak2 binding/activation and function. Mol Cell Biol 26: 8527–8536.
6. Malese K, Li F, Chong ZZ (2005) New avenues of exploration for erythropoiesis. JAMA 293: 90–95.
7. Celik M, Gokmen N, Erbayraktar S, Akhisaroğlu M, Konakc S, et al. (2002) Erythropoietin prevents motor neuron apoptosis and neurologic disability in experimental spinal cord ischemic injury. Proc Natl Acad Sci USA 99: 2258–2263.
8. Madeddu P, Emmanueli C (2007) Switching on Reparative Angiogenesis: Essential Role of the Vascular Erythropoietin Receptor. Circ Res 100: 599–601.
9. Sinclair AM, Coxon A, McCaffrey I, Kaufman S, Paveletz KL, et al. (2010) Functional erythropoietin receptor is undetectable in endotelial, cardiac, neuronal and renal cells. Blood DOI 10.1182/blood-2009-10-248666.
10. Divoxy V, Liu Z, Ryan TM, Prchal JF, Townes TM, et al. (2001) Mouse model of congenital polycythemia: homologous replacement of murine gene by mutant human erythropoietin receptor gene. Proc Natl Acad Sci USA 98: 986–991.
11. Zhang H, Sato K, Nakajima H, McKay C, Ney PA, et al. (2001) The distal region and receptor tyrosines of the Epo receptor are non-essential for in vivo erythropoiesis. EMBO J 20: 3156–3166.
12. Yu X, Shacka JJ, Eells JB, Suarez-Qian C, Przygodzki RM, et al. (2002) Erythropoietin receptor signalling is required for normal brain development. Development 129: 505–516.
13. Farrell F, Lee A (2004) The erythropoietin receptor and its expression in tumor cells and other tissues. The Oncologist 9(Suppl 5): 18–30.
14. Elliott S, Busse L, Bass MB, Lu H, Sarosi I, et al. (2006) Anti-Epo receptor therapy: a survival study. J Clin Oncol 23: 5960–5972.
15. Yu X, Shacka JJ, Eells JB, Przygodzki RM, et al. (2002) In vitro mass production of human erythroid cells from the blood of normal donors and of thalassemic patients. Blood Cells Mol Dis 28: 169–180.
16. Kralovics R, Sokol L, Prchal JT (1998) Absence of polycythemia in a child with a unique erythropoietin receptor mutation in a family with autosomal dominant primary polycythemia. J Clin Invest 102: 124–129.
17. Furukawa T, Narita M, Sakane M, Onuka T, Kuroha T, et al. (1997) Primary familial polycythemia associated with a novel point mutation in the erythropoietin receptor. Br J Haematol 99: 222–227.
18. Watowich SS, Xie X, Klingmuller U, Kere J, Lindlof M, et al. (1999) Erythropoietin receptor mutations associated with familial erythrocytosis cause hypersensitivity to erythropoietin in the heterozygous state. Blood 94: 2530–2532.
19. Rives S, Pahl HL, Florensa L, Bellosillo B, Neusser S, et al. (2007) Molecular genetic analyses in familial and sporadic congenital primary erythrocytosis. Haematologica 92: 674–677.
20. Al-Shemli M, Mazuzier E, Bardie C, Casadevall N, Galactèros F, et al. (2008) A study of 36 unrelated cases with pure erythrocytosis revealed three new mutations in the erythropoietin receptor gene. Haematologica 93: 1072–1075.
21. Prchal JT (2003) Classification and molecular biology of polycythemia (erythrocytoses) and thrombosis. Hematol Oncol Clin North Am 17: 1151–1158.
22. Bourantas UK, Chatziyiakakou A, Dassoula M, Bourantas KL, Georgiou I, et al. (2006) Absence of mutations of the EPO-receptor gene in Greek patients with familial polycythemia. Eur J Haematol 76: 537–538.
23. Queisser W, Heim ME, Schmitz JM, Worst P (1980) Idiopathic familial polycythaemia. Dtsch Med Wochenschr 113: 851–855.
24. Prchal JT, Senzena GE, Prchal J, Sokol L (1995) Familial polycythemia. Science 260: 1831–1833.
25. Van Maecken T, Hunnekink T, Callewaert L, Benoit Y, Laureys G, et al. (2004) Familial and congenital polycythemas: a diagnostic approach. J Pediatr Hematol Oncol 26: 407–416.
26. Migliazzo G, Di Pietro D, Di Balsarre A, Migliazzo AR, et al. (2002) In vitro mass production of human erythroid cells from the blood of normal donors and of thalassemic patients. Blood Cells Mol Dis 28: 169–180.
27. Bertolini F, Shaked Y, Mancuso P, Kerebel RS (2006) The multifaceted circulating endothelial cell in cancer: towards marker and target identification. Nat Rev Cancer 6: 835–845.
28. Delia Ragone F, Cucciololo V, Borriello A, Oliva A, Ferrotta S (2007) Erythropoietin receptors on cancer cells: a still open question. J Clin Oncol 25: 1812–1813.
29. Waldfan P, Verder F, Kadi Z, Chariot S, Lacombe C, et al. (2005) Both pronaseo and lysosomes degrade the activated erythropoietin receptor. Blood 105: 600–608.
30. Meyer L, Deau B, Foreijnková H, Dumitra D, Margotin-Goguet F, et al. (2007) β-Trcp mediates ubiquitination and degradation of the erythropoietin receptor and cells proliferation. Blood 109: 5215–5222.
36. Sulahian R, Cleaver O, Huang LJ, Sulahian R, Cleaver O (2009) Ligand-induced EpoR internalization is mediated by JAK2 and p85 and is impaired by mutations responsible for primary familial and congenital polycythemia. Blood 113: 5287–5297.

37. Li B, Jia N, Kapur R, Chun KT (2006) Cul4A targets p27 for degradation and regulates proliferation, cell cycle exit, and differentiation during erythropoiesis. Blood 107: 4291–4299.

38. Wu H, Lee SH, Gao J, Liu X, Iruela-Arispe ML (1999) Inactivation of erythropoietin leads to defects in cardiac morphogenesis. Development 126: 3597–3605.

39. Oppliger Leibundgut E, Horn MP, Brunold C, Pfanner-Mayer B, Marti D, et al. (2006) Hematopoietic and endothelial progenitor cell trafficking in patients with myeloproliferative diseases. Haematologica 91: 1463–1472.

40. Ronzoni L, Bonara P, Rusconi D, Fragomeni C, Libani I, et al. (2008) Erythroid differentiation and maturation from peripheral CD34+ cells in liquid culture: Cellular and molecular characterization. Blood Cells Mol Dis 40: 148–155.

41. Scott LM, Tong W, Levine RL, Scott MA, Beer PA, et al. (2007) JAK2 exon 12 mutations in polycythemia vera and idiopathic erythrocytosis. N Engl J Med 356: 459–468.

42. Cacciolla V, Borriello A, Criscuolo M, Sinisi AA, Bencivena D, et al. (2008) Histone deacetylase inhibitors upregulate p57Kip2 level by enhancing its expression through Sp1 transcription factor. Carcinogenesis 29: 560–567.

43. Borriello A, Cacciolla V, Criscuolo M, Indaco S, Oliva A, et al. (2006) Retinoic acid induces p27Kip1 Nuclear accumulation by modulate its phosphorylation. Cancer Res 66: 4240–4248.