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Reactivation of Hepatic EPO Synthesis in Mice After PHD Loss

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The hormone erythropoietin (EPO) promotes red blood cell production. The liver is the major source of EPO during embryogenesis. After birth, however, the kidney assumes this role, and hepatic EPO production is largely silenced. This switch has profound medical implications. For example, 20 million Americans have renal disease, of whom 10 to 20% are anemic. Treatment with pharmacological doses of recombinant EPO is costly, requires parenteral administration, and has been linked to cardiovascular side effects and increased mortality (1).

EPO transcription is regulated by HIF (hypoxia-inducible factor). The HIF α subunit is normally prolyl hydroxylated by a PHD (prolyl hydroxylase domain) protein, leading to its polyubiquitination by the von Hippel–Lindau protein (pVHL) and subsequent degradation. Under hypoxic conditions, however, PHD activity decreases and HIF α accumulates. HIF α , bound to HIF β , transcriptionally activates genes such as *EPO*. The canonical HIF family members, HIF1 α and HIF1 β , and most HIF target genes are ubiquitously expressed. In contrast, expression of *EPO* and HIF2 α , which regulates *EPO* in both the liver and kidney, is more restricted.

PHD2 is the primary HIF α prolyl hydroxylase and is required for embryonic development (2). Silencing PHD2 in adult mice causes increased renal, but not hepatic, *EPO* production and polycythemia (overproduction of red blood cells) (3, 4). PHD1 and PHD3 are nonessential genes that fine-tune the HIF response (5).

The PHDs belong to a superfamily of ~70 2-oxoglutarate-dependent dioxygenases (2). These enzymes can be inhibited with druglike molecules, some of which induce hepatic *EPO* synthesis in anephric mice (6). Conceivably this reflects their ability to inactivate the PHDs. However, inactivation of other 2-oxoglutarate-dependent di-oxygenases, including enzymes that affect chromatin, might also be involved.

To determine whether PHD loss is sufficient to reactivate hepatic *EPO* production, we created mice with livers lacking PHD1, PHD2, PHD3, or combinations thereof by using null (*Phd1* and *Phd3*) and floxed (*Phd2*) alleles (5). *Phd2* or, as a control, *Vhl*, was inactivated by using Cre recombinase driven by a hepatocyte-specific transgene (7). Hepatic inactivation of PHD1, PHD2, or PHD3 alone did not increase *EPO* or hematocrit values (Fig. 1, A and B, and fig. S1). Consistent with previous data (3), hematocrits were modestly

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Supporting Online Material

www.sciencemag.org/cgi/content/full/329/5990/407/DC1

Materials and Methods

Fig. S1

References

increased in mice lacking PHD1 and PHD3 (Fig. 1B and fig. S1). Loss of all three PHDs, however, dramatically increased EPO and hematocrit values (Fig. 1, A and B, and fig. S1). Indeed, these EPO concentrations vastly exceeded those achieved after renal PHD2 inactivation (4, 5, 7).

HIF α concentrations, particularly HIF2 α concentrations, were conspicuously elevated in livers lacking pVHL or all three PHDs, suggesting that exceeding a threshold amount of HIF2 α is sufficient to drive hepatic *Epo* expression (Fig. 1, A and B). The development of polycythemia in mice expressing a stabilized version of HIF2 α in the liver supports this idea (8). Although hepatic *Vhl* inactivation causes benign tumors (7), we have not observed tumors in PHD-defective mice.

The albumin promoter that we used to express *Cre* becomes active during late embryogenesis, before completion of the switch from the liver to the kidney as the main EPO source, raising the possibility that PHD inactivation can prevent, but not reverse, silencing of the hepatic *Epo* locus. To address this, we inactivated *Phd2* in 4- to 8-week-old *Phd1*^{-/-}; *Phd2* *flox/flox*; *Phd3*^{-/-} mice by using a ubiquitously expressed Cre–estrogen receptor fusion protein, which was activated by tamoxifen (Fig. 1C) or by tail vein injection of an adenovirus encoding green fluorescent protein (GFP)–Cre (fig. S1). Both interventions reactivated hepatic EPO synthesis (Fig. 1C and fig. S1). These effects were specific because they were not seen in *Phd2*^{+/+} mice or in mice given an adenovirus encoding GFP alone (fig. S1).

These findings, together with earlier work, suggest that PHD2 inactivation is sufficient to induce near maximal renal EPO production whereas inactivation of all three PHDs is needed to reactivate hepatic EPO production. Thus, drugs that inhibit all three PHDs will probably be required to treat anemia linked to chronic renal failure, whereas drugs that inhibit PHD2 alone might suffice in nephric patients.

Certain features of HIF agonists suggest that they may be safer than pharmacological doses of EPO. For example, HIF activates a suite of genes dedicated to erythropoiesis and might therefore promote red blood cell production at lower circulating EPO concentrations. Moreover, HIF activates genes, such as inducible nitric oxide synthase, that might lower blood pressure and cardiovascular risk. Whether any of these theoretical benefits are real remains to be determined in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References and Notes

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Fig. 1.

Hepatic EPO synthesis. **(A)** Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis of hepatic *Epo* mRNA (solid bars) and serum Epo concentrations (open bars) in 4-week-old male mice with indicated genotypes. $n = 3$ mice per group. Pound symbols indicate $P < 0.0001$ comparing indicated genotype versus wild type (wt). DKO, double knockout; TKO, triple knockout. Note that *Phd2* KO and *Vhl* KO are liver-specific. Error bars indicate 1 SEM. **(B)** Immunoblots of livers from 4-week-old mice as in (A). Mean hematocrits (Hct) are also shown. **(C)** Real-time RT-PCR analysis of hepatic *Epo* mRNA from 4-week-old mice with indicated genotypes 1 week after tamoxifen treatment. $n = 3$. $**P < 0.01$. Error bars indicate 1 SEM.