

## Hypoxia-inducible erythropoietin expression: details matter

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Recombinant human erythropoietin (rhEPO) and its derivatives are some of the most important treatment options against anemia associated with chronic kidney disease, chemotherapy, and low-risk myelodysplastic syndrome.<sup>1</sup> On the other hand, it has also gained fame as a doping agent in endurance sports. All this became possible by translating basic research findings that started more than 100 years ago with the recognition of an increase in red blood cell number in animals living at high altitude (i.e., at a decreased pO<sub>2</sub>) and that went, *via* purification, partial sequencing, cloning, and basic understanding of *EPO* gene regulation, into clinics (for excellent reviews of EPO history see<sup>2-5</sup>).

With its cloning and the fast-improving molecular biological tools, findings from hypoxia-inducible *EPO* gene regulation also became a paradigm to understand basic principles of how cells sense and adapt to oxygen availability. The discovery of those principles were acknowledged by part of the 2019 Nobel prize in Physiology or Medicine.<sup>6</sup>

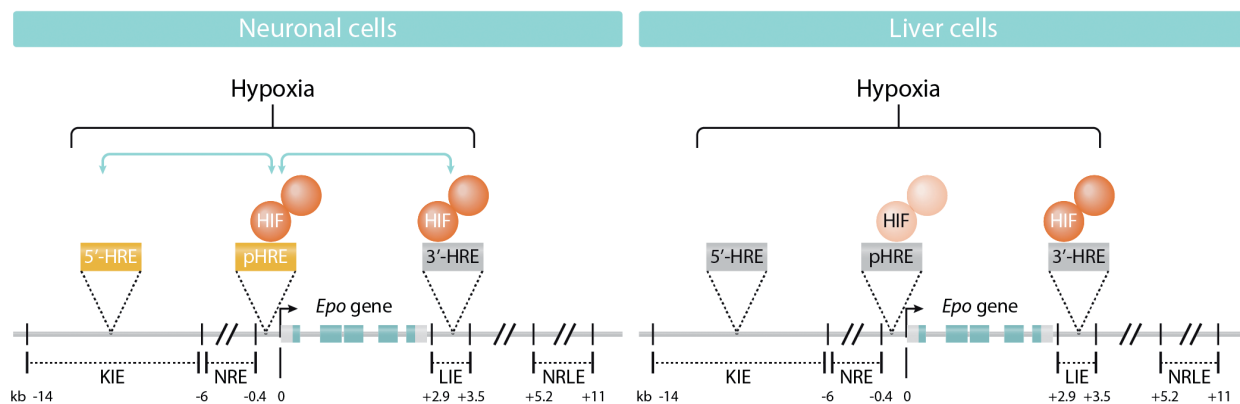
The major production sites of EPO are peritubular fibroblasts in the kidney as well as hepatocytes and hepatic stellate cells (about 90% of circulating EPO stems from kidney and 10% from liver). But EPO expression can also be found in brain, testis, uterus, and osteoblasts.<sup>7-10</sup> Apart from its major function as a driver of erythropoiesis, EPO was also found to exert organ- and tissue-restricted protective functions in the brain, cardiovascular system, adipose tissue, and bones.<sup>11,12</sup>

Experiments with transgenic mice revealed that critical parts of the *EPO* gene that are located away from the proper EPO coding exons in the distal 5'- and 3'-flanking

regions are responsible for tissue-specific and hypoxia-driven *EPO* gene expression. Up to then, it had been known that an array consisting of the kidney-inducible element (KIE; between -14 kb to -9.5 kb 5' from the promoter) and a negative regulatory element (NRE, between -6kb to -0.4kb) in the 5'-part seem to be of special importance for EPO expression in kidney (Figure 1). By contrast, in liver, hypoxia-inducible EPO expression appeared to depend largely on an enhancer in the so-called liver-inducible element (LIE) that was found 3'-distal from the *EPO* polyadenylation side (Figure 1). That area contained, among other functional sites, an HRE (hypoxia response element) that served as a binding site for a hypoxia-inducible factor (HIF).<sup>13-16</sup> From the three HIFs known, HIF-2 $\alpha$  has been defined as a major part of the hypoxia-inducible *EPO* gene activating complex.<sup>17,18</sup>

While the role of the HRE in the 3'-enhancer is well established and found to be necessary and sufficient to confer liver-specific *EPO* gene expression, the DNA element responsible for kidney-specific *EPO* gene expression is far less well characterized and nothing was known about the presence and function of HREs in EPO-producing none-kidney tissues such as neurons of the brain. This problem has been tackled by Orlando and co-workers, and the outcome of their studies is described in an article in the current issue of *Haematologica*.<sup>19</sup>

In their studies, the authors built on findings where they discovered a distal 5'-HRE within a DNaseI hypersensitive site -9.2 kb upstream of the *EPO* transcriptional start site which was supposed to contribute to oxygen-regulated *EPO* expression in the kidney.<sup>20</sup> As the EPO-producing cells in the kidney derive from neural crest and



**Figure 1. Hypoxia-regulated human erythropoietin (*EPO*) gene expression in neuronal and liver cells.** Hypoxia leads to stabilization of hypoxia-inducible factor (HIF), and mainly HIF-2 containing complexes are able to induce *EPO* gene expression. In neuronal cells, HIF-containing complexes bind most strongly to the newly identified hypoxia response element (HRE) in the promoter and to a lesser extent to the 3'-HRE. Although HIF-complexes appear not to bind the 5'-HRE, the 5'-HRE co-operates in the hypoxic induction process. In liver cells, HIF-complexes bind mainly to the 3'-HRE, weakly to the pHRE, and not to the 5'-HRE. Lighter colors indicate less HIF binding; green arrows indicate co-operation between the HREs. KIE: kidney-inducible element; NRE: negative regulatory element; LIE: liver-inducible element; HRE: hypoxia response element; NRLE: negative regulatory liver element; Prom: promoter; pHRE: promoter HRE functioning as one entity but consisting of a tandem dimeric repeat with two HRE sequences.

neuroepithelial cells,<sup>21</sup> the authors went on to compare the function of this part containing the 5' HRE and the established 3'-HRE in human neuroblastoma cells and liver hepatocellular carcinoma cells; both cell types are able to express EPO in response to hypoxia.<sup>22</sup>

To do this, they used gene editing by CRISPR/Cas9 to specifically mutate the -9.2 kb 5'-HRE and the +3.0 kb 3'-HRE in the *EPO* gene locus, and combined this with subsequent HIF-DNA interaction studies, RNA and protein expression measurements and reporter gene assays.

Intriguingly, when analyzing *EPO* mRNA and secreted *EPO* protein levels upon exposure of the engineered cells to hypoxia (0.2% O<sub>2</sub>) for 24 hours, the authors found that the neuroblastoma cells with mutations in both the 5'-HRE and the 3'-HRE did not increase *EPO* mRNA and protein levels to the same extent as wild-type cells. By contrast, only mutation of the 3'-HRE reduced the hypoxic *EPO* induction in hepatoma cells.

When the authors expanded these experiments by transiently transfecting cells with minimal *EPO* promoter-driven reporter genes, enhanced by various DNA fragments containing the 5'- and/or 3'-HRE, they found that *EPO* promoter-driven reporter gene activity in neuroblastoma cells was best when a 100 bp 5'-region containing the HRE was combined with a 126 bp HRE containing 3'-fragment.

Further experiments with different *EPO* promoter HRE constructs indicated that the most efficient hypoxia-dependent *EPO* induction requires co-operation between the *EPO* 5'- and 3'-HRE specifically in neuronal cells, and that additional distal and proximal 5'-flanking elements further contribute to tissue-specific and conditional *EPO* regulation.

In addition, the minimal *EPO* gene promoter alone was able to promote hypoxia-dependent reporter gene activation in neuroblastoma cells and hepatoma cells indicating that this part may contain previously unidentified HRE. Indeed, the authors found a tandem dimeric repeat with two HRE sequences that had not previously been reported. Importantly, mutation of each single HRE reduced reporter gene activity, but simultaneous mutation of both HREs did not have an additional effect, suggesting that both HREs behave as an entity.

Next, the authors were able to link the observed findings to HIF binding. Chromatin immunoprecipitation assays in neuroblastoma cells revealed that none of the HIF subunits bound to the 5'-HRE, whereas a significant hypoxic increase in HIF-2 $\alpha$ /HIF- $\beta$  binding to the 3'-HRE could be detected. By contrast, in hepatocellular carcinoma cells, HIF-2 $\alpha$ /HIF- $\beta$  binding could be detected with the promoter region and the 3'-HRE. Importantly, non-*EPO* producing cells did not show any HIF binding to the examined regions of the *EPO* gene. Further experiments with the CRISPR/Cas9 engineered cells showed that mutation of the 3'-HRE also decreased HIF binding to the promoter region; vice versa, mutation of the 5'-HRE impaired HIF binding to the 3'-HRE.

Taken together, the study shows that hypoxic *EPO* expression in hepatic cells appears only to depend on HIF interaction with the 3'-HRE and not on the 5'-HRE or the HIF binding *EPO* promoter. By contrast, neuronal *EPO* expression seems to require co-operation with intact 5'-

and 3'-HRE, with HIF interacting strongly with the new HRE in the *EPO* promoter, and to a lesser extent with the 3' HRE.

Another important aspect of this study is that hypoxia-dependent gene expression is not a uniform process and needs to be seen and analyzed in a cell type-specific context. This is because some cells' HIF may not bind to all HREs, although they may confer oxygen sensitivity. This may be of importance in gene-wide association studies where different SNPs or disease-associated polymorphisms may represent HREs which may, depending on the context, gain or lack their function.

Overall, the investigation by Orlando *et al.*<sup>19</sup> presents novel and interesting findings highlighting that a complex interplay between various HREs, HIFs and other factors at the *EPO* locus contribute to its hypoxia-dependent and organ-specific expression.

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## COVID-19: risk of infection is high, independently of ABO blood group

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When a French aircraft carrier set sail on 22 January, 2020 for a mission of several months, its 1,769 crewmembers were unaware of a stowaway in the form of a novel virus. The SARS-CoV-2 virus, assumed in early 2020 to be a recent arrival in Europe, was already on board. Upon the ship's return to Toulon, the main naval base of France on the Mediterranean Sea, most of the crew were confined to their barracks and 1,688 sailors participated in health monitoring. In this issue of *Haematologica*, Boudin and colleagues report data from this unique epidemiological setting,<sup>1</sup> which could have hardly been better designed, if it had been set up for the purpose of studying a SARS-CoV-2 outbreak among young professionals.

After 1 month at sea, the first case of COVID-19 was recognized. Another month went by before an epidemic broke out, which forced the ship's early return to base within 2 weeks. Several viral strains were detected by nucleotide sequencing.<sup>1</sup> This observation could imply the embarkation of multiple sailors who were independently infected, an unlikely scenario in Europe so early in the pandemic. Possibly, only one crewmember was the source, and the initial strain evolved within the 12 weeks' voyage while spreading among the crew.

Due to its exponential rate of spread, the SARS-CoV-2 virus rapidly infected at least 1,279 sailors, 76% of the participants of the study, whose median age was 28 years. Only 13% were female, without difference in the infection rate between males and females.<sup>1,2</sup> This rate seemed strikingly high among young, healthy individuals,<sup>1</sup> although it may not differ so much from that of other SARS-CoV-2 outbreaks, but rather reflected an exceptionally thorough follow-up and documentation. Only 14% of the infected participants remained asymptomatic.<sup>1</sup> The median age of the 19 patients requiring only oxygen therapy was 45 years; the five patients admitted to intensive care units were older than 50 years. All infected sailors recovered eventually. These relatively benign clinical courses may not be representative of COVID-19 among the general population or cruise ship passengers, with a decidedly older age profile and related comorbidities.<sup>3</sup>

A PubMed search for "ABO in COVID-19" yielded more than 50 publications including reviews and meta-analyses,<sup>4,5</sup> documenting this possible correlation as a topic of intense research in the past 9 months.<sup>5,6</sup> The study by Boudin *et al.*<sup>1</sup> provides data leading to an important clarification: the rate of infection among young adults is independent of ABO blood group. This study can be considered the definitive conclusion on this aspect, as the quality of the epidemiological data was optimal. Studies in smaller cohorts<sup>7-10</sup> and less well-defined epidemiological settings<sup>7-9,11</sup> should be considered with caution, even if there are many. They are more likely to be affected by unknown cofounders. Particular precaution should be applied when COVID-19 was associated with ABO along with other blood group systems.<sup>10</sup> Better data on ABO blood group and SARS-CoV-2 infection may not be accrued soon, and any future study would have to measure up to the quality of the study by Boudin *et al.*<sup>1</sup> Can the ABO in COVID-19 topic be considered settled?

An early study did not claim an influence of ABO on the SARS-CoV-2 infection rate.<sup>12</sup> Rather the clinical course and disease outcome in patients, once infected, may differ depending on the ABO blood group.<sup>3,6,7,9,12,13</sup> The lack of convincing evidence for an association between ABO and outcome in some,<sup>10,14</sup> even many, studies cannot be construed as convincing evidence for lack of such an association. The largest and most comprehensive data set so far was from patients with respiratory failure.<sup>15</sup>

This genome-wide association study<sup>15</sup> reported a small association signal coinciding with the chromosomal position of the ABO blood group system. Outcome was better for patients with blood group O than for those with blood group A. The study design was criticized for using blood donors as the majority of controls.<sup>14</sup> Using flawed control cohorts is a notorious cause of erroneous conclusions,<sup>16</sup> and blood donors are generally selected in favor of blood group O.<sup>17</sup> However, it remains to be explored whether the odds ratio introduced by this well-founded bias of Spanish<sup>6</sup> and Italian donor recruitment, could entirely explain the odds ratio of excess death associated with blood group A.<sup>15</sup> Even