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TOPICAL REVIEW

Erythropoietic and non-erythropoietic functions of erythropoietin in mouse models

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Abstract As the basic function of erythropoietin (Epo) is stimulation of red blood cell production, systemic overexpression of Epo results in erythrocytosis. The patho-physiological consequences of chronically elevated red blood cell counts have been studied in Epo overexpressing mice. Genetically modified mice, however, have also played an important role in discovering multiple additional functions of Epo besides stimulating erythrocyte production. Non-erythropoietic functions of Epo are widespread and play a role in organogenesis during early embryonic development and in tissue protection in ischaemic diseases. Future work in the field will most likely focus on these additional functions of Epo, which have great clinical potential.

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Introduction

Detailed phenotyping of experimentally mutated mice is helpful in understanding physiology. Thus, it was a milestone in understanding biological processes and the multiple interactions between the different organ systems of the mammalian organism when genetically modified mice became available for research. This era started in 1977 with the first transgenic mouse (Jaenisch, 1977) and the refinement of this technique a few years later (Palmiter & Brinster, 1985). Finally, the discoveries of how homologous recombination between segments of DNA molecules can be used to specifically target genes in the mammalian genome was honoured by the 2007 Nobel Prize in Physiology or Medicine to Drs Mario R. Capecchi, Martin J. Evans and Oliver Smithies. In this review we will summarize the insights into the biology of erythropoietin (Epo) gained from the investigation of genetically modified mice regarding Epo or its receptor (EpoR).

Epo-trangenic mice

The first Epo-transgenic mouse line harbouring the full-length human Epo gene was published 1989 (Semenza *et al.* 1989), and was followed up by additional ones from the same group (Semenza *et al.* 1991). These mouse lines were used to study the mechanisms resulting in tissue specific expression of Epo in liver and kidney. Analogously, another group generated several different Epo-transgenic mouse lines in order to study the effects of various amounts of flanking DNA on Epo gene expression (Madan *et al.* 1995). The next Epo-transgenic mouse model published (Ruschitzka *et al.* 2000) was generated to assess the cardiovascular effects of excessive erythrocytosis. Most recently, a transgenic mouse line expressing the human Epo cDNA under the control of the bovine β-casein promotor has

Johannes Vogel (left) and **Max Gassmann** (right) do their research at the University of Zurich in the Institute of Veterinary Physiology and the Zurich Center for Integrative Human Physiology (ZIHP). Their scientific interests are cardiovascular physiology, the molecular and systemic response to hypoxia, the multiple non-erythropoietic functions of erythropoietin (Epo), and the function of the calcitonin receptor-like receptor and its ligands. Their research is based mainly on *in vivo* studies by analysing the different and often unexpected phenotypes of genetically modified mice.

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Table 1. Some non-erythropoietic functions of Epo as discovered using genetically modified mice

Mouse model	Main finding	Reference
$Epo^{-/-}$ or $EpoR^{-/-}$	Embryonically lethal due to anaemia and heart hypoplasia.	(Wu *et al.* 1999)
EpoR-tg or EpoR-LacZ-tg	EpoR is highly expressed in the embryonic brain.	(Liu *et al.* 1997)
Brain restricted Epo-tg (tg21)	Brain-derived Epo (brainstem) regulates the hypoxic ventilatory response	(Soliz *et al.* 2005)
tg6 and tg21	Epo is protective in cerebral infarction as long as the haematocrit is normal	(Wiessner *et al.* 2001)
tg6	Systemic Epo is protective in hypoxia induced pulmonary hypertension	(Weissmann *et al.* 2005)
tg6, rd1 & VPP	Epo is protective in light-induced photoreceptor apoptosis but not retinitis pigmentosa	(Grimm *et al.* 2004)
Cardiomyocyte-specific sonic $hedgehog^{-/-}$	Cardioprotective effects of Epo in heart ischaemia require sonic hedgehog signalling	(Ueda *et al.* 2010)

Additional non-erythropoietic Epo functions have been established also in wild-type animals as well as cell culture models (cf. text).

been generated but only one out of five founding lines survived exhibiting a moderate erythrocytosis with a haematocrit of 63% (Kim *et al.* 2007). In particular, the so-called tg6 mouse line generated by Ruschitzka *et al.* (2000) has been used extensively to study the pathophysiology of excessive erythrocytosis, but in the past decades it turned out that Epo has many other functions apart from promoting red cell production.

Non-erythropoietic functions of Epo

In addition to the studies on the pathophysiological consequences of excessive erythrocytosis, other genetically modified mouse lines have revealed important insights into Epo's additional biological effects that were independent of the production of red cells (Table 1). This started with studies on mice lacking either Epo or its receptor (EpoR) (Wu *et al.* 1999). Mice deficient for Epo or EpoR die around embryonic day 13.5 only partly due to impaired erythropoiesis. In the developing mouse heart, EpoR is expressed between embryonic day 10.5 and 14.5. The loss of Epo signalling in the embryonic heart results in severe ventricular hypoplasia independent of hypoxia due to a reduction in the number of proliferating cardiac myocytes as well as epicardium detachment and abnormalities in the vascular network (Wu *et al.* 1999).

Epo signalling might also play a role in the development of the central nervous system. Different experiments analysing binding of ^{125}I-labelled Epo, EpoR mRNA levels as well as the expression of lacZ under control of the EpoR promotor have revealed that EpoR is highly expressed in the embryonic brain and also to a lesser extent in the adult central nervous system (Digicaylioglu *et al.* 1995; Marti *et al.* 1996; Liu *et al.* 1997; Chen *et al.* 2006). In addition, progenitor cells from endothelial and skeletal muscle show EpoR expression (Anagnostou *et al.* 1994; Ogilvie *et al.* 2000). In this context it is interesting that lethality of EpoR deficiency can be rescued by EpoR expression restricted to erythroid cells, resulting in mice without obvious phenotype (Suzuki *et al.* 2002). This finding indicates that further studies are necessary to define the function of Epo in normal embryonic development. In adulthood, however, the lack of EpoR in non-haematopoietic tissues might be a disadvantage in disease situations, as has been demonstrated by rescuing EpoR deficient mice (Suzuki *et al.* 2002) for pulmonary hypertension (Satoh *et al.* 2006). In line with these findings, tg6 mice that chronically over-express the human Epo gene do not develop pulmonary hypertension in normoxia or after exposure to chronic hypoxia (10% O_2 for 3 weeks) (Weissmann *et al.* 2005).

Despite the fact that the EpoR is massively (4 orders of magnitude) down-regulated in neuronal tissues until embryonic day 17 (Liu *et al.* 1994), there is considerable evidence that Epo is functional in the adult brain. For example, acclimatization to a hypoxic environmant involves carotid body stimulation by peripherally produced Epo as well as Epo up-regulation in combination with down-regulation of the soluble EpoR in central respiratory centres of the brainstem (Soliz *et al.* 2005, 2007). Of great clinical importance is that Epo is supposed to have protective functions in many different tissues (Sasaki *et al.* 2001). In the brain, both Epo and its receptor are up-regulated during ischaemia/hypoxia (Sakanaka *et al.* 1998; Siren *et al.* 2001*b*) and Epo administration considerably inhibits apoptosis after middle cerebral artery occlusion (Siren

et al. 2001*a*). These findings resulted in a clinical study in stroke patients confirming the beneficial effects of Epo therapy in case of brain ischaemia (Ehrenreich *et al.* 2002) but only in patients not additionally receiving recombinant tissue plasminogen activator (Ehrenreich *et al.* 2009). In line with this, in mice overexpressing Epo solely in the brain (termed tg21) infarct volume was about 22% but not significantly lower compared to wild-type controls (Wiessner *et al.* 2001). In contrast, the same study showed that mice systemically overexpressing Epo with haematocrit values around 0.85 (tg6) had 49% enlarged infarct volumes suggesting that increased haematocrit levels and/or the concomitantly elevated serum NO levels might reduce possible protective effects of Epo after stroke. Apart from its positive effects in acute ischaemic brain damage, Epo was also shown to be beneficial for treatment of blunt cortical trauma, neurotoxin exposure and experimental autoimmune encephalitis (a model of multiple sclerosis) (Brines *et al.* 2000). In keeping with neuronal tissue, the mouse retina is protected against light-induced degeneration by inhibiting photoreceptor cell apoptosis by Epo, induced by either hypoxic preconditioning or direct application of recombinant human Epo (Grimm *et al.* 2002). Similarly, Epo overexpression in the retina as it occurs in tg6 mice protects against light-induced retinal degeneration (Grimm *et al.* 2004). Photoreceptor apoptosis is also the common path in retinitis pigmentosa (RP). Crossbreeding with two mouse models of human RP with tg6 mice, however, did not affect the time course or the extent of retinal degeneration in a light-independent (rd1) and a light-accelerated (VPP) mouse model of RP. Similarly, repetitive intraperitoneal injections of recombinant human Epo did not protect the retina in the rd1 and the VPP mouse. These effects were not due to adaptational downregulation of Epo receptor (Grimm *et al.* 2004). Thus, Epo appears to be protective in the retina during acute, light-induced photoreceptor cell death but not in genetically based retinal degeneration.

Apart from its beneficial effects under pathological conditions, Epo appears to modulate cognitive function. This effect can at least partly be explained by changes in haematocrit (Weiskopf *et al.* 2006), but there is most likely an additional effect of Epo directly in the brain. In contrast to brain ischaemia, in healthy mice systemically administered Epo needs to cross the blood–brain barrier to exert effects on neurons. Indeed, it was suggested that that 0.5–1% of a systemically administered high Epo dosage crosses undamaged the blood–brain barrier, according to the authors, by receptor mediated transcytosis (Brines *et al.* 2000; Brines & Cerami, 2005). Intraperitoneal injections of Epo in healthy wild-type mice improved sequential learning and memory components of a complex long-term cognitive task (El-Kordi *et al.* 2009). This can be explained by modulating plasticity, synaptic connectivity and activity of memory-related neuronal networks in the hippocampus (Adamcio *et al.* 2008). Effects of Epo in brain tissue of healthy mice require Epo's capability to cross the intact blood–brain barrier. As Epo is a large molecule (molecular mas: 34 kDa) this appears to be quite unlikely and consequently has been questioned (Boado *et al.* 2010).

Similar to ischaemic neuronal tissue, Epo has tissue protective effects also in other tissues suggesting a stereotypic mechanism, maybe with the endothelial cells as a key player in this process. Indeed, the endothelium was the first non-haematopoietic tissue described as a physiological target for Epo (Knudtzon & Mortensen, 1975). Later it was established that Epo is a growth and chemotactic factor for endothelial cells (Anagnostou *et al.* 1990). Moreover tissue repair appear to be at least supported by Epo-induced release of endothelial progenitor cells from the bone marrow (Heeschen *et al.* 2003; Westenbrink *et al.* 2007; Santhanam *et al.* 2008). Numerous studies show also the expression of Epo receptors on other than endothelial cells (Noguchi *et al.* 2008). For example in the ischaemic heart this effect appears to rely on direct protective effects on cardiomyocytes and endothelial cells as well as stimulation of angiogenesis (Ruifrok *et al.* 2008). Another example is the protective effect of stimulation of non-erythropoietic Epo receptors observed in a murine kidney ischaemia–reperfusion model (Brines *et al.* 2008). The protective properties of Epo might rest on a counterbalancing effect of Epo against tumour necrosis factor α (TNFα) that ultimately results in increased apoptosis of cell that escaped the initial tissue damage (Brines, 2010). In addition, Epo appears to promote tissue repair by supporting or inducing angiogenesis. Mice studies revealed that the transcription factor sonic hedgehog could play a role in promoting the pro-angionetic effects of Epo in the post-ischaemic heart (Ueda *et al.* 2010).

The non-erythropoietic functions of Epo might be transduced by a receptor distinct of that found on erythroid cells. Interestingly it could be shown that on erythroid cells interleukin 3 (IL3) also is able to stimulate haemoglobin synthesis (DiFalco & Congote, 2002). While on erythroid cells the EpoR is a homodimer (Jelkmann *et al.* 2008), the IL3 receptor is a heterodimer consisting of an α-subunit with high affinity to IL3 and the common βc-subunit. It has been speculated that in extra-haematopoietic tissues the Epo receptor is a heterodimer with one Epo receptor monomer and the common βc-subunit that is shared by the IL3, IL5 and GM-CSF receptors (Leist *et al.* 2004; Brines & Cerami, 2006). These distinct Epo receptors could represent the physiological basis for development of tissue protective Epo analogons that have no unwanted effects on haematopoiesis.

Taken together there is overwhelming data showing that Epo has many non-erythropoietic functions. As such, it

appears incomprehensible that some authors still claim that the EpoR has no other biological function than erythropoiesis (Sinclair *et al.* 2010; Swift *et al.* 2010), as this statement has been clearly refuted (Ghezzi *et al.* 2010).

Conclusion and outlook

In summary, genetically modified mice have taught us that Epo has several non-haematopoietic functions. This was a clinically very important discovery and will surely be the future direction of the research in the field. Thus, many new details about these additional functions of Epo will be discovered as expression of Epo, and its receptor, has been shown in numerous tissues including the reproductive organs, gastric mucosal cells, pancreatic islets, inner ear and prostate epithelial cells. The exact mechanisms for the non-erythropoietic functions of Epo await further elucidation but there are efforts to develop drugs that specifically stimulate Epo receptors in non-erythropoietic tissues, thus avoiding the negative side effects of increased haematocrit values (Ueba *et al.* 2010). The rationale behind these efforts is based on the fact the EpoR in non-erythopoietic tissues might differ from that transducing erythropoiesis (Masuda *et al.* 1993). Accordingly, peptide analogues of the B-helix of the four α-helix (A–D)-containing Epo molecule were successfully used to induce tissue protection without increasing haematocrit (Leist *et al.* 2004; Brines *et al.* 2008). One day these peptides might be replaced by small, orally active non-erythopoietic Epo receptor mimetics.

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