Renal anemia: from incurable to curable

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Sato Y, Yanagita M. Renal anemia: from incurable to curable. Am J Physiol Renal Physiol 305: F1239–F1248, 2013. First published July 24, 2013; doi:10.1152/ajprenal.00233.2013.—Renal anemia has been recognized as a characteristic complication of chronic kidney disease. Although many factors are involved in renal anemia, the predominant cause of renal anemia is a relative deficiency in erythropoietin (EPO) production. To date, exogenous recombinant human (rh)EPO has been widely used as a powerful drug for the treatment of patients with renal anemia. Despite its clinical effectiveness, a potential risk for increased mortality has been suggested in patients who receive rhEPO, in addition to the economic burden of rhEPO administration. The induction of endogenous EPO is another therapeutic approach that might have advantages over rhEPO administration. However, the physiological and pathophysiological regulation of EPO are not fully understood, and this lack of understanding has hindered the development of an endogenous EPO inducer. In this review, we will discuss the current treatment for renal anemia and its drawbacks, provide an overview of EPO regulation in healthy and diseased conditions, and propose future directions for therapeutic trials that more directly target the underlying pathophysiology of renal anemia.

The management of renal anemia has been revolutionized since the development of recombinant human erythropoietin (rhEPO). Before the advent of rhEPO, severe anemia was a common complication in uremic individuals (45), resulting in disabling fatigue, diminished quality of life, and the need for frequent blood transfusions, which are potentially hazardous. In 1977, EPO was first purified by Miyake and colleagues (86). They spent several years collecting urine from patients with aplastic anemia, and, from this urine, they obtained a small quantity of pure glycoprotein, which enabled determination of the protein sequence and cloning of its cDNA (72). In the late 1980s, rhEPO became available for clinical use, which revolutionized the management of renal anemia. Correction of anemia with rhEPO has dramatically improved the debilitating symptoms and diminished the need for red blood cell transfusions (22, 28). In addition, numerous beneficial effects of rhEPO therapy, including the regression of left ventricular hypertrophy (78, 110) and improved cognitive function (80), have been reported.

However, adverse effects of rhEPO, such as hypertension and thrombotic complications, have also been reported (60, 128). In addition, a recent randomized trial of anemia correction using rhEPO in patients with chronic kidney disease (CKD) and end-stage renal disease (ESRD) failed to demonstrate a benefit of higher hemoglobin levels and suggested hazardous effects of EPO (25, 96, 111, 112). Although the mechanisms responsible for the poor outcome observed in these clinical trials were not fully elucidated, these negative results require that we reconsider optimal treatment strategies for patients with renal anemia.

In this review, we will discuss the current treatment for renal anemia and provide an overview of EPO regulation in healthy and diseased conditions. We will also propose future directions for therapeutic trials that more directly target the underlying pathophysiology of renal anemia.

Current Treatment for Renal Anemia

Erythropoiesis-stimulating agents (ESAs) have been used to treat patients with renal anemia. ESAs can be classified into two categories depending on their mechanisms of action (Fig. 1): one mechanism of ESA action is exogenous administration of rhEPO or its peptide mimetics, such as Hematide, and another mechanism involves inducers of endogenous EPO production.

To date, exogenous rhEPO administration has been shown to effectively improve and maintain hemoglobin levels and has become the standard treatment for renal anemia. Furthermore, in vivo and in vitro studies (10, 98) have shown that EPO possesses organ-protective effects that are independent from its hematopoietic effects. For example, exogenous EPO administration before or after injury is highly effective for the prevention of ischemic or hypoxia-induced injury in multiple organs and tissue (8, 11, 14, 51). Despite promising results in experimental animals, however, there is growing evidence suggesting a potential risk of increased mortality in patients who receive exogenous rhEPO (25, 96, 111, 112).

Endogenous EPO induction by several mechanisms (as discussed in Novel Therapeutic Approaches for Renal Anemia) is considered to have several potential advantages over rhEPO administration, including better availability, lower immunogenicity (18, 33, 119), ease of administration, greater stability at room temperature (76), and the potential coordinative induction of other genes that are important for erythropoiesis (43, 52).
ESRD. For example, renal anemia has been shown to worsen an absolute loss of EPO-producing cells (82, 92). There are universal in patients with ESRD (2, 30). It has long been a hypothesis that EPO-producing cells lose their ability to produce EPO as renal function is the main cause of renal anemia. Thus, an endogenous EPO inducer must be developed.

One plausible explanation for the lack of EPO inducer development is an incomplete understanding of kidney-specific EPO regulation and the behavior of EPO-producing cells in both physiological and pathophysiological conditions. The elucidation of EPO-producing cell behavior and EPO regulation has been hindered by several obstacles. First, there has been a lack of appropriate cell lines that represent EPO-producing cells in the kidney, which has hampered the determination of molecular EPO regulatory mechanisms in the kidney (43, 117). In 1987, Goldberg et al. (37) identified two human hepatoma cell lines displaying oxygen-dependent EPO expression: HepG2 and Hep3B. Most of the present knowledge of the oxygen-sensing mechanism that controls EPO production is based on the findings derived from in vitro studies using these nonrenal EPO-producing cells (117, 121). Caution is required when these findings in hepatic cells are applied to EPO-producing cells in the kidney, because the regulation of EPO expression is tissue specific (121). Frede et al. (31) were the first group to establish a cell line derived from human kidney that expresses EPO in an oxygen-dependent manner. This cell line was isolated from tumor-free tissue of a male patient with renal carcinoma and will become a powerful model to study the molecular mechanisms of renal EPO expression. Second, an appropriate method to trace EPO-producing cells in the kidney while EPO production is reduced has also been lacking, which has compromised the investigation of EPO-producing cells. Obara et al. (93) established bacterial artificial chromosome (BAC) transgenic mouse lines that express green fluorescent protein (GFP) as a reporter under the control of a 180-kb genomic region containing the human EPO gene. GFP signals are turned off.

The transition of the EPO production site. EPO is essential for erythropoiesis, particularly for later stages of erythroid differentiation. Mice with disrupted EPO or EPO receptor genes cannot develop erythropoiesis and die from anemia in utero (71, 122). While many hematopoietic growth factors are produced by bone marrow cells in the vicinity of their target cells, EPO is produced outside the bone marrow, mainly in the kidney and liver. The relative contribution of the kidney and liver to the production of EPO is still debated.

Physiology of EPO

As kidney disease progresses, the relative EPO deficiency becomes more frequent, and this deficiency becomes almost universal in patients with ESRD (2, 30). It has long been a subject of investigation and debate whether the relative EPO deficiency in patients with CKD is a functional disturbance or an absolute loss of EPO-producing cells (82, 92). There are epidemiological data and clinical observations suggesting that EPO-producing cells exist in the kidneys of patients with ESRD. For example, renal anemia has been shown to worsen after bilateral nephrectomy, which was previously performed to control blood pressure in dialysis patients (56), suggesting that EPO production is retained to some extent even in the kidneys of patients with ESRD. Several observational studies (9, 16) have also shown that plasma EPO concentrations in dialysis patients could be increased in response to acute episodes of hypoxia and hemorrhage. More recently, Brookhart et al. (13) investigated whether higher altitude affects either the requirement for rhEPO administration or hematocrit levels among a large cohort of hemodialysis patients in the United States. They demonstrated that higher altitude was associated with elevated hematocrit and a reduced need for rhEPO. Compared with patients at sea level, patients living above 6,000 ft received 19% less rhEPO but still had higher hematocrit (35.7% vs. 34.6%). This result indicates the presence of an EPO feedback control even in patients with ESRD.

Other clinical evidence showing that EPO-producing cells exist even in the kidneys of patients with ESRD came from the results of a clinical trial. EPO gene expression is induced in response to hypoxic insults (26), which is mediated mainly by hypoxia-inducible factor (HIF). HIFs facilitate adaptation to oxygen deprivation by regulating the expression of gene products that are important for angiogenesis, erythropoiesis, and anaerobic glucose metabolism (35, 105). HIFs are heterodimeric proteins consisting of an α-subunit and a β-subunit. HIF α-subunits are regulated by oxygen tension at the protein level, whereas β-subunits are constitutively expressed. Thus, HIF transcriptional activity is determined by the hypoxic stabilization of its α-subunits. In normoxia, α-subunits are rapidly hydroxylated by a family of prolyl hydroxylase domain proteins (PHDs), which initiate HIF-α proteasomal degradation. PHD inhibitors stabilize HIF-α and activate HIF transcriptional activity independent of oxygen availability. Bernhardt et al. (7) conducted a phase 1 clinical trial of a PHD inhibitor (FG-2216) and demonstrated that pharmacological stabilization of the HIF system increases EPO production, even in patients with ESRD. Plasma EPO levels in patients with ESRD that received FG-2216 became 30.8-fold higher than those of patients with ESRD patients that did not receive FG-2216. This suggested that dysfunction of EPO-producing cells, rather than death of the cells, may cause the inappropriately low EPO production in patients with ESRD.

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liver to EPO production is primarily age dependent (43, 113). During mammalian development, erythropoiesis moves from the fetal liver to the bone marrow and spleen (36, 95). In parallel with this transition, the predominant EPO production site also switches from the liver to the kidney. Interestingly, the manner of EPO action is different between the liver and the kidney (113). EPO secreted from the fetal and neonatal liver stimulates erythropoiesis in a paracrine manner, whereas EPO secreted from the kidney functions on erythroid cells in the bone marrow and spleen in an endocrine manner. Although there is a difference between species in the timing of the transition and relative contribution of the liver or kidney to EPO production, the transition of the EPO production site has been confirmed in both animals and humans, and it usually occurs during late gestation or approximately at birth (21, 89).

There are several hypotheses to explain the reason why the mammalian kidney, a nonhematopoietic organ, produces EPO in adult life. One possible reason is that there is hypoxia in the kidney, which renders the kidney more sensitive to subtle changes in oxygen delivery compared with other tissues, and stimulates EPO production (91). A second explanation regards the kidney as a criptimeter (24). Hematocrit is determined by plasma volume and red blood cell mass. The kidney might have the ability to set the hematocrit within a normal range by coordinating red blood cell mass through EPO secretion and by regulating plasma volume. Another explanation is that the kidney is evolutionally an erythropoietic tissue; erythropoietic tissue is found in the kidney of most teleosts, chondrosteans, and holosteans (92).

**EPO-producing cells in the kidney and their characteristics.** The site of EPO production in the kidney has been controversial for quite some time. Although there have been many attempts to determine the location of EPO production in the animal kidney, the results have been inconsistent (63, 64, 75), partly due to low EPO expression under normoxic condition. The requirement to maintain a steady state of endogenous EPO in a healthy person is very small, and renal EPO expression can be detected only with very sensitive methods, such as RT-PCR.

Studies in different types of transgenic mice have also failed to find conclusive evidence on the location of EPO-producing cells. Constitutive and ectopic expression of the transgene have been frequently detected in several transgenic reporter mouse studies using constructs containing \(<20 \text{ kb of the EPO gene}-flanking region (75, 79, 107, 108). These results suggested that regulatory elements are important for EPO gene expression. As mentioned above in Current Treatment for Renal Anemia, Obara et al. used the BAC vector, incorporating a large genomic fragment, to generate a transgenic mouse line (68) and successfully visualized EPO expression using GFP (93). Using this BAC transgenic mouse line, these researchers demonstrated that interstitial fibroblasts in the kidney are the EPO-producing cells.

Under normoxic conditions, there are few EPO-producing cells, and these are restricted only in the deep cortex and outer medulla of the kidney. Under hypoxic conditions, such as in severe anemia, however, the number of EPO-producing cells increases exponentially, and EPO-producing cells spread outward from deep cortex toward the capsule (59, 93). Studies using in situ hybridization and the transgenic mouse approach showed that the EPO signal intensity in each EPO-producing cell is comparable. These observations suggest that renal EPO production is regulated mainly by the number of EPO-producing cells rather than by EPO gene expression levels in individual EPO-producing cells (59, 93). Although it is still unknown if EPO-producing cells proliferate or if there is an increase in the number of cells that produce EPO, which exceeds the detection threshold under hypoxic conditions, the latter seems to be more plausible than the former suggestion.

EPO-producing cells also have neural characteristics. They have dendrite-like processes and express the neuronal markers, such as microtubule-associated protein 2 and neurofilament light polypeptide (93). Another group (31) has also confirmed the expression of these two markers in EPO-producing cells in vitro.

**Determinants of the serum EPO level.** The plasma EPO level depends on the balance between the rate of EPO production and its removal from the circulation. In contrast to other hormones such as insulin, which is stored within the cell until a secretory stimulus, EPO is secreted just after it is produced. Thus, the rate of EPO secretion reflects the rate of EPO production, which, in turn, is primarily determined by EPO gene expression in kidneys (for a discussion, see Refs. 2–4). However, secreted EPO is degraded in the body, and the degradation products are excreted in urine (85). Gross et al. (40) demonstrated that degradation of EPO occurs in cells expressing the EPO receptor. After EPO binds to its receptor, it is internalized by endocytosis and then degraded in lysosomes (40).

**Regulation of EPO gene expression.** EPO production is controlled at the transcriptional level. The EPO gene promoter is suppressed by GATA transcriptional factors in normoxia (50), whereas the enhancer is activated by HIFs in hypoxia (42). The cell type-specific regulation of these transcriptional factors allows unique characteristics of EPO gene expression: tissue-specific and hypoxia-inducible regulation. Although several tissues have been reported to express the EPO gene (29), the kidney accounts for \(~90\%\) of total EPO production in the adult human (57, 58).

Tissue-specific EPO gene expression is a result of the GATA-induced inhibition of EPO expression in renal tubular epithelial cells and in other tissues (93). Obara et al. showed that a single-nucleotide mutation in the promoter GATA box reactivates EPO expression in epithelial cells of various tissues, such as the thymus, bile duct, bronchus, and tubular epithelial cells of the kidney. In addition, tumors that originated from renal tubular epithelial cells occasionally produce EPO and cause polycythemia in humans (104). Recently, Kurt and colleagues (62) demonstrated that von Hippel-Lindau (VHL) protein, a negative regulator of HIF, also plays important role in the inhibition of EPO expression in juxtaglomerular cells in the kidney. They showed that, using the Cre/loxP system, deletion of VHL converts renin-producing cells into EPO-producing cells.

On the other hand, the adaptation of EPO production to oxygen supply is determined mainly by HIF. HIF is composed of an oxygen-sensitive \(\alpha\)-subunit and a constitutive \(\beta\)-subunit. Under normoxia, \(\alpha\)-subunits are rapidly hydroxylated by a family of PHDs. Hydroxylation of \(\alpha\)-subunits allows capture by the VHL tumor suppressor gene product, ubiquitination, and destruction by the proteasome. However, under hypoxic conditions, when there is a low level of oxygen molecules, HIF would not be hydroxylated and is thereby stabilized. Accumulated HIF binds to the key sequence of the EPO enhancer, the...
hypoxia-responsive element, and activates the transcription of EPO. Several studies have demonstrated that subtle changes in blood oxygen content stimulate EPO secretion. For example, EPO secretion is normally inversely regulated by the hemoglobin level (1), establishing an efficient feedback control of red blood cell production.

To date, three different HIF α-isoforms have been identified: HIF-1α, HIF-2α, and HIF-3α (43, 87, 117). Although these isoforms share several characteristics, such as an oxygen-dependent hydroxylation and DNA-binding sequence, their localization and functionality are different. HIF-1α is expressed ubiquitously, whereas HIF-2α and HIF-3α are expressed in a more restricted way (69). In addition, HIF-1 and HIF-2 heterodimerize to function as transcriptional activators, whereas splice variants of HIF-3α seem to function as suppressors of other HIFs (43, 69, 117). Although HIF-1 and HIF-2 share many transcriptional targets, they do not overlap completely. For example, genes regulating anaerobic glycolysis are predominantly controlled by HIF-1 (48), whereas EPO production and genes for iron metabolism are regulated by HIF-2 (41, 52, 81, 109). The different localization and function of HIF α-subunits may allow the regulation of oxygen-dependent genes in distinct cell-specific and tissue-specific manners.

Both HIF-1α and HIF-2α are expressed in the adult kidney. Several lines of experimental evidence have shown that HIF-2α, rather than HIF-1α, is the crucial factor that drives EPO transcription. The most convincing evidence for this theory comes from conditional knockout studies in mice. Since HIF-1α and HIF-2α knockout mice are embryonic lethal (99), Gruber and colleagues (41) developed a mouse model with inducible inactivation of HIF-α using the tamoxifen-CreERT2 system and investigated the physiological role of HIF-1α and HIF-2α in the adult (41). In this model, conditional deletion of HIF-2α resulted in anemia, whereas conditional deletion of HIF-1α did not alter hematocrit. In addition, these researchers demonstrated that, under hypoxia, activation of EPO transcription was substantially reduced in HIF-2α conditional knockout mice, whereas conditional knockout of HIF-1α did not interfere with EPO induction during hypoxia. A gain-of-function mutation in the HIF-2α gene has been also reported to be responsible for excessive erythrocytosis in humans (100). Moreover, Kapitsinou et al. (52) ablated renal HIF-2α using a Cre-loxP recombination, and they showed that hypoxic induction of EPO in the kidney exclusively depends on HIF-2α; they also showed that in the absence of renal HIF-2α, hepatic HIF-2α becomes the main regulator of the serum EPO pool.

Paliege and colleagues (94) offered anatomic insights regarding the distribution of HIF-1α, HIF-2α, and EPO in the rat kidney. They used two models to mimic hypoxic conditions, oxygen deprivation and HIF stabilization with a PHD inhibitor, and demonstrated that HIF-2α and EPO are expressed in the same renal interstitial cells, whereas HIF-1α is predominantly expressed in renal tubular cells. These results are consistent with those of previous reports that suggested that HIF-2α, rather than HIF-1α, is the crucial factor driving EPO transcription.

The liver is the main source of extrarenal EPO production, although the EPO-producing capacity of the liver is much lower than that of the kidney in adult animals (27, 32, 116). Similar to the kidney, the liver responds to hypoxia by increasing the number of EPO-producing hepatocyte around the central vein (93). Oral administration of a PHD inhibitor to anephric dialysis patients slightly increased serum EPO levels, although the induction was weaker compared with nephric dialysis patients, suggesting that hepatic EPO synthesis were induced by systemic HIF stabilization and contributed to the serum EPO pool (7). Interestingly, hepatocyte-derived HIF-2α is also involved in the regulation of iron metabolism (52).

Decline of EPO production in the diseased kidney. In general, anemia becomes more frequent as renal function declines, becoming almost universal in patients with ESRD (4, 47, 55). Hsu and coworkers (47) conducted a cross-sectional study of 12,055 adult ambulatory patients and found that mean hematocrit values decreased progressively when creatinine clearance was below 60 ml/min in men and below 40 ml/min in women. This sex difference diminished when the renal function measurement was indexed to body size. Kohagura and coworkers (55) also reported similar results in Japanese populations.

Serum EPO levels in patients with CKD are generally normal or slightly increased, which is considered to be inappropriately low relative to the degree of anemia, because serum EPO levels in similarly anemic patients with normal kidney function are 10–100 times higher than the normal range (20, 34, 83). Fehr and coworkers (30) studied 395 patients randomly chosen from patients who underwent coronary angiography and showed an altered set point for EPO production in patients with renal dysfunction. Although hemoglobin levels negatively regulate serum EPO levels in patients with creatinine clearance above 40 ml/min, serum EPO levels in patients with creatinine clearance below 40 ml/min did not increase despite the lower hemoglobin levels in these patients.

Interestingly, hemoglobin levels in some patients on hemodialysis are maintained without rhEPO therapy or blood transfusion. Goodkin and colleagues (38) analyzed data from the Dialysis Outcomes and Practice Patterns Study and demonstrated the presence of a subgroup whose hemoglobin concentrations were >12 g/dl in the absence of rhEPO administration; they called these patients “endogenous EPO” patients. Endogenous EPO patients tended to be male, to have a longer duration of ESRD, and not to dialyze using a catheter. In addition, cystic disease as a cause of ESRD was associated with a markedly increased likelihood of having endogenous EPO status. Together with the evidence showing that higher altitude is associated with higher hematocrit levels and a lower requirement for rhEPO in patients with ESRD (13), these results suggest that patients with CKD are diverse and heterogeneous populations, which makes it difficult to use the universal protocol management of renal anemia.

Origin and Behavior of EPO-Producing Cells

Although the essential role of EPO has been well recognized, the behavior and developmental origin of EPO-producing cells remain unknown, which has hindered both the generation of EPO-producing cells from stem cells as well as clarification of the molecular mechanisms underlying the defective production of EPO in patients with CKD.

In 1974, Le Douarin et al. (67) performed xenotransplantation of a quail neural tube into a chick embryo and demonstrated that neural crest-derived cells contribute to the developing kidneys. Similar results were obtained with a dye injection experiment into the dorsal neural tube (12, 103). The neural crest is a unique and transient structure in the vertebrate
embryo. It arises from the neural tube and migrates to many embryonic tissues, where the cells differentiate into a variety of cell types, such as smooth muscle cells, melanocytes, peripheral glial cells, and neurons (66). Recent reports have indicated that neural crest cells also contribute to bone marrow cells (90) and mesenchymal stem cells (88).

Interestingly, neural crest-derived cells and EPO-producing cells in the kidney share several common characteristics; both are HIF-2α-positive cells (41, 118) that have a neuron-like phenotype (93). Based on these observations, we hypothesized that neural crest-derived cells migrate into the embryonic kidney and differentiate into interstitial fibroblasts, including EPO-producing cells, during embryogenesis. To test this hypothesis, we conducted a lineage tracing study (3) that used myelin protein zero (P0)-Cre lineagenegative mice (P0-Cre mice), which express Cre recombinase in the neural crest (3). P0-Cre lineage-labeled cells were located in the kidney interstitium and expressed platelet-derived growth factor receptor (PDGFR)-β and CD73/ecto-5′-nucleotidase (5′-NT), suggesting that P0 lineage gave rise to interstitial fibroblasts. More than 98% of interstitial fibroblasts in the cortex and outer medulla were lineage labeled with P0-Cre. Among these fibroblasts, <10% of fibroblasts produced EPO even under severely anemic condition. Within this population, ~80% of EPO-producing cells were lineage labeled with P0-Cre.

**EPO-producing cells transdifferentiate into myofibroblasts.** In the diseased kidney, α-smooth muscle actin (α-SMA)-positive myofibroblasts are generally accepted as the key effector cells in the pathogenesis of fibrosis. Despite its biological importance, however, the origin of myofibroblasts has long been a subject of investigation and debate (for reviews, see Refs. 39, 61, 101, and 126). Until recently, epithelial mesenchymal transition (EMT) has been hypothesized, we conducted a lineage tracing study (3) that used myelin protein zero (P0)-Cre transgenic mice (P0-Cre mice), which express Cre recombinase in the neural crest (3). P0-Cre lineage-labeled cells were located in the kidney interstitium and expressed platelet-derived growth factor receptor (PDGFR)-β and CD73/ecto-5′-nucleotidase (5′-NT), suggesting that P0 lineage gave rise to interstitial fibroblasts. More than 98% of interstitial fibroblasts in the cortex and outer medulla were lineage labeled with P0-Cre. Among these fibroblasts, <10% of fibroblasts produced EPO even under severely anemic condition. Within this population, ~80% of EPO-producing cells were lineage labeled with P0-Cre.

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**Myofibroblasts retain functional plasticity and can be a therapeutic target in patients with CKD.** We proposed the transdifferentiation of renal EPO-producing cells into myofibroblasts as a potential mechanism by which EPO-producing cells lose their ability to synthesize EPO during the progression of CKD (3). Our work also emphasizes that dysfunction of neural crest-derived cells in the kidney is the cause of renal anemia and fibrosis. Targeting neural crest-derived cells might represent a new therapeutic approach not only to specifically suppress the generation of α-SMA-positive myofibroblasts but also to restore EPO production. Because EPO-producing cells in the
kidney possess both renal and neural characteristics, we administered renoprotective and neuroprotective agents to cultured primary myofibroblasts obtained from fibrotic kidneys to determine whether or not the EPO-producing ability could be restored. Notably, we found that the attenuated EPO production could be restored by the administration of neuroprotective agents such as low-dose dexamethasone and neurotrophins and the renoprotective agent HGF. In addition, we also found that the administration of tamoxifen, a selective estrogen receptor modulator (SERM), reversed the attenuated EPO expression and fibrosis in fibrotic kidneys in vivo. These results suggest that myofibroblasts retain functional plasticity and can be a therapeutic target in patients with advanced CKD.

**Novel Therapeutic Approaches for Renal Anemia**

The upcoming therapeutic agents and strategies for stimulating erythropoiesis and treating anemia have resulted from the determination of the molecular mechanisms that control erythropoiesis. The development of effective therapies for renal anemia has been a highly active field, and there are several reviews on this topic (for reviews, see Refs. 76 and 77).

As discussed above, recent randomized controlled clinical trials have raised significant safety concerns on the overuse of EPO, as suggested in Federal Drug Administration black box warnings, which might be related to poorer outcomes. To circumvent the adverse effects of rhEPO therapy, a more desirable approach must be the reversal of the phenotype of myofibroblasts into healthy fibroblasts, which allow the kidney to produce necessary and sufficient amount of native EPO in response to hypoxia. Here, we discuss an endogenous EPO inducer and novel possible therapeutic strategies supported by our recent findings.

**PHD inhibitors.** As discussed previously, PHD inhibitors stabilize HIF-α, activate HIF transcriptional activity independent of oxygen availability, and increase EPO transcriptional activity. Recently, an additional EPO-independent role for HIF-2α in erythropoiesis has received much attention. For example, HIF-2α plays a critical role in promoting intestinal absorption of iron (81, 109) and regulating the hematopoietic microenvironment (123), which is essential for effective erythropoiesis. Iron deficiency, which causes EPO resistance, is common both in CKD and dialysis patients (5). Thus, compared with ESA alone, pharmacological activation of HIF may provide an advantage by coordinating the induction of other genes that are important for erythropoiesis. The effectiveness of PHD inhibitors has already been demonstrated in animal models and humans (7, 46, 52, 65, 102).

Unfortunately, however, the activation of HIF has multiple downstream effects, and intermittent HIF activation over a prolonged period of time may lead to profound changes in cellular metabolism, growth, and differentiation (42). In addition, during one of the phase II clinical trials with FG-2216, a PHD inhibitor, a female patient developed fatal hepatic necrosis that was temporally related to the introduction of this compound (76). Thus, there are many hurdles to overcome before this reagent could be introduced in a clinical setting.

Recently, Querbes et al. (102) succeed in specifically targeting PHDs in the liver and were able to reactivate hepatic EPO synthesis by knocking down PHDs with small interfering RNA packed in lipid nanoparticles. While hypoxia-induced EPO production in the liver does not compensate for renal anemia, pharmacological stabilization of hepatic HIF can be sufficient to correct anemia characterized by absolute or relative deficiency of EPO (52, 102). Compared with systemic administration of PHD inhibitors, this may have the advantage of avoiding any effects on HIF target genes in other tissues.

**Neurotrophin, HGF, and glucocorticoids.** As discussed above, we have shown that the administration of neurotrophins, HGF, and glucocorticoids restored the attenuated production of EPO in myofibroblasts (3). Neurotrophins have been reported to promote neuronal growth and survival in many neuronal populations through the activation of Trk receptor tyrosine kinases (17, 97, 120). In addition, neurotrophin-3 rescues neuronal precursors from apoptosis and promotes neuronal differentiation in embryonic kidney explants (53). Given the origin of EPO-producing cells from P0-Cre-expressing precursors, it is plausible that neurotrophins restore the EPO-producing ability in transdifferentiated myofibroblasts (3).

Recently, a proof-of-principle study (102) established pharmaceutical strategies to stimulate neurotrophin signaling as effective therapeutic agents for patients with Alzheimer’s disease. Among these strategies, small-molecule activators of neurotrophin receptors are potential candidates and should be tested using the model of renal anemia in the future.

Another possibility is glucocorticoids, which exhibit both protective and destructive effects in the nervous system. In excess, glucocorticoids produce neuronal death, whereas low-dose glucocorticoids are neuroprotective. Recently, it has been demonstrated that glucocorticoids provide a neuroprotective effect through the activation of neurotrophin signaling. However, glucocorticoids have numerous adverse effects on many organ systems, which are more common in patients who receive these drugs over a long period of time. In this point of view, the benefit of long-term glucocorticoid therapy for renal anemia seems to be counterbalanced by its adverse effects.

In addition, the beneficial effect of HGF has been reported in regenerating kidney injury (74). The regenerative capacity of HGF may be effective in restoring the EPO-producing ability in myofibroblasts.

**SERMs.** It is widely recognized that women have a slower progression of nondiabetic kidney disease compared with men (44). Estrogen is one of the candidates responsible for sex differences in the progression of kidney disease. In animal models, estrogen and SERMs have been reported to be renoprotective (6, 19, 23, 127). SERMs activate estrogen receptors and function as estrogen agonists in some tissues, such as the bone and kidneys, and as estrogen antagonists in other tissues, such as the breast. Recently, raloxifene, a SERM, was reported to be renoprotective in postmenopausal women with osteoporosis (84). We (3) have also demonstrated SERMs can reverse the phenotype of myofibroblasts into healthy fibroblasts. This approach might be desirable because this cannot be accompanied with the undesirable adverse effects of other ESAs, such as toxicity of excess rhEPO and multiple HIF-regulated biological responses, including angiogenesis. Further clarification of the mechanism of action and clinical trials of SERMs used to treat patients with CKD with renal anemia are needed.

**Regenerative medicine: mesenchymal stem cells and induced pluripotent stem cells.** Generating functional transplantable tissue from autologous stem cells is a goal in regenerative medicine. Yokoo et al. (125) generated a human mesenchymal
stem cell (hMSCs)-derived organoid that was morphologically and functionally similar to the kidney. They cultivated hMSCs in growing rodent embryonic kidneys and showed that hMSCs contributed to functional structures of the embryonic kidney. Using this culture system, they succeeded in generating an EPO-producing organoid derived from hMSCs in which EPO production was stimulated by the induction of anemia (124). In addition, they demonstrated that the serum levels of EPO generated in rats by the organoid were sufficient to treat renal anemia.

On the other hand, induced pluripotent stem (iPS) cells have also received great interest in regenerative medicine because of their multilineage differentiation potential and easy to use in vitro expansion (114, 115). Our findings that EPO-producing cells are derived from neural crest cells might open a new avenue of research toward the in vitro generation of EPO-producing cells from iPS cells. This is promising because an efficient method for converting iPS cells to neural crest cells has already been established (15).

Conclusions

The advent of rhEPO, which improved patients’ quality of life by reducing unpleasant symptoms and the need for blood transfusions, has been a major advance in the care of patients with kidney disease within the last three decades. This history truly demonstrates the clinical relevance of basic research in this field. The research in this field has progressed toward a more precise understanding of the biology of EPO-producing cells. Future studies will help to obtain a better understanding of renal anemia and eventually a safer and more efficient therapy for patients with this condition.

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AUTHOR CONTRIBUTIONS

Author contributions: Y.S. and M.Y. conception and design of research; Y.S. and M.Y. interpreted results of experiments; Y.S. and M.Y. prepared figures; Y.S. and M.Y. drafted manuscript; Y.S. and M.Y. edited and revised manuscript; Y.S. and M.Y. interpreted results of experiments; Y.S. and M.Y. prepared figures; M.Y. analyzed data.

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