Review

Crosstalk between oxygen signaling and iron metabolism in renal interstitial fibroblasts

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To maintain the oxygen supply, the production of red blood cells (erythrocytes) is promoted under low-oxygen conditions (hypoxia). Oxygen is carried by hemoglobin in erythrocytes, in which the majority of the essential element iron in the body is contained. Because iron metabolism is strictly controlled in a semi-closed recycling system to protect cells from oxidative stress caused by iron, hypoxia-inducible erythropoiesis is closely coordinated by regulatory systems that mobilize stored iron for hemoglobin synthesis. The erythroid growth factor erythropoietin (EPO) is mainly secreted by interstitial fibroblasts in the renal cortex, which are known as renal EPO-producing (REP) cells, and promotes erythropoiesis and iron mobilization. Intriguingly, EPO production is strongly induced by hypoxia through iron-dependent pathways in REP cells. Here, we summarize recent studies on the network mechanisms linking hypoxia-inducible EPO production, erythropoiesis and iron metabolism. Additionally, we introduce disease mechanisms related to disorders in the network mediated by REP cell functions. Furthermore, we propose future studies regarding the application of renal cells derived from the urine of kidney disease patients to investigate the molecular pathology of chronic kidney disease and develop precise and personalized medicine for kidney disease.

Key Words: **erythropoietin, iron mobilization, renal anemia, renal fibrosis, urinary exfoliated cells**

When the first organism emerged, oxygen concentrations were very low on Earth, and the evolution of early organisms progressed away from the harmful reactivity of oxygen to biomolecules.[1,2] After the appearance of aerobic organisms that could use oxygen for energy production while detoxifying oxygen, oxygen has driven the explosion of evolution by providing metabolic systems that are effective for energy production. Inevitably, aerobic organisms must incessantly consume oxygen, which cells cannot store.[3,4]

For proteins or cells, iron is a suitable molecule for maintaining and releasing oxygen in a rapid response to changing metabolic conditions. However, because iron is cytotoxic, aerobic cells and organisms need to strictly control iron metabolism and iron storage in a semi-closed system.[5] Thus, oxygen metabolism and iron metabolism in mammals are cooperatively and precisely regulated.[6] Approximately 70% of the iron in a human body is distributed in erythrocytes, in which 1 hemoglobin (Hb) molecule contains 4 iron atoms, to deliver oxygen to every peripheral organ. Erythropoiesis is closely associated with the mobilization of stored iron, which accounts for <30% of the total iron in the body.[5,6] This paper explains the molecular mechanisms by which oxygen and iron collaboratively regulate erythropoietin (EPO) production in renal interstitial fibroblasts and the mechanism by which EPO simultaneously promotes erythroid cell differentiation and iron mobilization.

Iron is Associated with Hypoxia-Inducible EPO Production in REP Cells

In adult mammals, EPO is mainly produced and secreted by interstitial fibroblasts distributed in the renal cortex, and these cells are referred to as renal EPO-producing (REP) cells.[6–11] Therefore, kidney injury or nephrectomy often cause EPO-deficiency anemia, which is known as renal anemia. Under hypoxic conditions caused by factors such as bleeding, high-altitude areas, and respiratory diseases, plasma concentrations of EPO are dramatically increased to maintain the erythrocyte-mediated oxygen supply to peripheral organs.[12] Hypoxia-inducible EPO production in REP cells is fundamentally controlled at the transcription level by hypoxia-inducible transcription factors (HIFs).

HIFs consist of a hypoxia-inducible α subunit and a constitutive β subunit. The β subunit is also known as aryl hydrocarbon receptor nuclear translocators (ARNTs), and there are 3 isoforms of HIF-α proteins encoded by independent genes.[13,14] Among the HIF-α isoforms, HIF2α is the major activator of *EPO* gene expression in REP cells (Fig. 1).[15,16] HIF2α is consistently synthesized in REP cells but immediately degraded by the proteasome under normal oxygen conditions (normoxia). Hydroxylation of the specific prolyl residues of HIF-α proteins, which is mediated by prolyl hydroxylase domain proteins [PHDs, also known as HIF-prolyl hydroxylases (HIF-PHs)], triggers their degradation.[13,14] Three PHDs have been identified in mammalian cells and commonly require molecular oxygen, ferrous iron (Fe^{2+}) and α-ketoglutarate for their catalytic activity (Fig. 1).[13,17] Under hypoxic conditions, oxygen is unavailable for PHDs, and HIF2α is subsequently stabilized and induces the transcription of its target genes by avoiding hydroxylation and degradation. Among the PHD isoforms, PHD2 dominantly controls EPO production in REP cells by sensing a lack of oxygen availability and catalyzing HIF2α hydroxylation (Fig. 1).[15,16]

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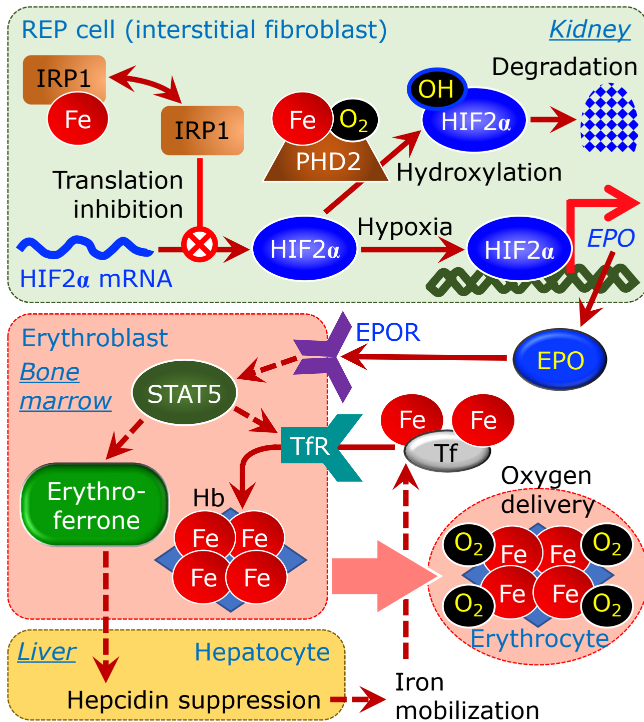

Fig. 1. Roles of oxygen and iron in erythropoietic regulation. EPO production is fundamentally controlled at the transcriptional level by HIF2α in a hypoxia-inducible manner in REP cells. HIF2α synthesis is blocked by IRP1 binding to HIF2α mRNA, which is inhibited by iron–sulfur clusters. Under normal oxygen conditions, HIF2α protein is constitutively degraded through PHD2-mediated hydroxylation of HIF2α, which requires ferrous iron and molecular oxygen (O_2). Under oxygen-depleted conditions (hypoxia), HIF2α is not degraded and induces the transcription of its target genes, including the *EPO* gene. EPO is secreted by REP cells and stimulates erythroferrone and TfR expression in erythroblasts through the EPOR-STAT5 signaling cascade. Erythroferrone suppresses hepatic production of hepcidin, a negative regulator of stored iron mobilization, and then erythroblasts take up transferrin (Tf)-iron from the blood through TfR for hemoglobin (Hb) synthesis. Mature erythrocytes deliver oxygen to every organ by using Hb.

Little is known about the mechanisms by which PHD2 and HIF2α among their isoforms, are involved in hypoxia-inducible *EPO* gene regulation in REP cells. Additionally, signaling pathways that control *EPO* gene expression exclusively in REP cells have not been identified. To elucidate the mechanisms of cell type-specific and hypoxia-inducible *EPO* gene expression, we analyzed *cis*-regulatory elements of the mouse *Epo* gene in reporter transgenic mice.[7,8,18–23] Although studies have demonstrated that the multiple regulatory elements involved in REP cell-specific and hypoxia-inducible *EPO* gene expression are located between 17 kb and 4 kb upstream of the transcription start site, the specific sequences within the upstream region and transcription factors other than HIF2α, which bind to *EPO* gene regulatory sequences, have still not been identified. Further studies using genome-wide single-cell techniques are needed to determine the molecular mechanisms of EPO production.[11]

EPO production is regulated in response to oxygen availability in REP cells, and iron is associated with *EPO* gene regulation at multiple steps. Iron deficiency blocks the translation of HIF2α, the main transcriptional activator of the *EPO* gene, through the induction of a physical interaction between iron-binding protein 1 (IRP1) and the 5' terminus of HIF2α mRNA, as well as translational regulation of other iron-regulatory proteins, such as ferritin chains and ferroportin (Fig. 1).[24] Under the iron-replete conditions, the iron–sulfur cluster binds to IRP1 and blocks its binding to HIF2α mRNA. Thus, EPO production is attenuated by a decrease in HIF2α activity when iron is unavailable for Hb synthesis and erythropoiesis. On the other hand, iron overload attenuates hypoxia-inducible HIF2α accumulation and *EPO* gene expression in REP cells.[25] Although the mechanisms by which iron negatively regulates HIF2α and EPO levels have yet to be elucidated, iron may enhance HIF2α degradation even under hypoxic conditions because ferrous iron is used for PHD-mediated HIF-prolyl hydroxylation (Fig. 1).[17,25,26]

EPO Induces Erythropoiesis by Triggering Erythroid Maturation and Systemic Iron Mobilization

After EPO is secreted from REP cells in the kidneys, EPO is delivered to the bone marrow, where it stimulates the maturation and proliferation of erythroblasts by binding to its specific receptor (EPOR). Since EPOR is more highly expressed on the surface of erythroid cells than on other cell types, EPO exclusively targets erythroid lineage cells.[6,27,28] One EPO molecule binds to an EPOR homodimer and alters the gene expression profile by activating various signaling cascades, and the Janus kinase 2 (JAK2)-signal transducer and activator of transcription 5 (STAT5) pathway plays a central role (Fig. 1).[6,28–30]

EPO stimulation induces the phosphorylation of STAT5, and activated STAT5 translocates into the nucleus from the cytoplasm to activate transcription of its target genes, which are related to anti-apoptotic effects and iron use for Hb synthesis.[6,28,30] The *TFRC* gene, which encodes the transferrin (Tf) receptor (TfR), is upregulated by STAT5 in erythroblasts receiving EPO and promotes the incorporation of holo-Tf containing ferric iron (Fig. 1).[6,20] EPO-EPOR signaling not only promotes iron use by erythroblasts but also impacts systemic iron metabolism by inducing erythroblastic expression of the *FAM132B* gene, another STAT5-target gene (Fig. 1).[6,31,32] The *FAM132B* gene product, erythroferrone, suppresses hepcidin production in hepatocytes after secretion from erythroblasts. Since hepcidin strongly inhibits ferroportin-mediated iron export from cells responsible for iron storage and iron absorption, erythroferrone induction provokes systemic iron availability to support Hb synthesis in erythroid cells (Fig. 1).

REP Cells are Transformed into Myofibroblasts, Thereby Promoting Renal Fibrosis

Chronic kidney disease (CKD) currently affects more than 10% of the global population. However, there is no treatment to cure CKD due to the unexplained molecular pathology of kidney disease, which is caused by various primary diseases and has complex prognosis.[33] Fibrosis is a final common pathway in complicated kidney disease, and myofibroblasts emerge and produce extracellular matrix.[34] REP cells are one of the most potent origins of the renal myofibroblasts.[35–39] We showed that oxidative stress in renal tubules is involved in the progression of kidney disease with fibrosis caused by the transformation of REP cells into myofibroblasts. Our previous report also demonstrated that activation of the antioxidative transcription factor Nrf2 in the earlier stages of kidney injury protects tubules from oxidative damage and suppresses renal fibrosis.[40,41] Thus, elucidating the molecular mechanism by which REP cells transform into myofibroblasts is expected to lead to the development of innovative therapies against renal fibrosis and CKD.

Analyses of a myofibroblastic cell line derived from murine REP cells (Replic cells) demonstrated that REP cells gain proliferative activity and produce extracellular matrix after undergoing fibroblast-to-myofibroblast transformation.[42,43] Additionally, renal EPO production after myofibroblastic transformation of REP cells is attenuated by constitutive degradation of HIF2α due to an unidentified mechanism which may make PHDs resistant

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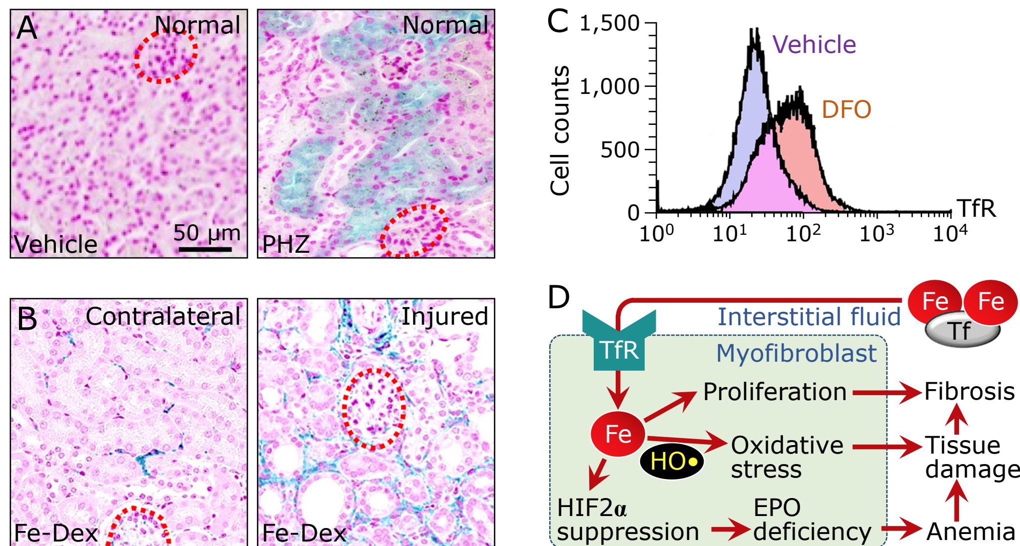

Fig. 2. Iron deposition in the interstitium of healthy and injured kidneys. (A) Berlin blue staining of kidney sections showing that phenylhydrazine (PHZ)-induced hemolytic anemia causes iron deposition (blue stain) in the tubular cells of mouse kidneys. (B) Iron dextran injection (Fe-Dex) induced iron deposition in the interstitial cells of the injured and contralateral kidneys of mice subjected to unilateral ischemia–reperfusion injury (uIRI). PHZ (50 mg/kg body weight) or vehicle (saline) was intraperitoneally injected into the mice daily for 3 days, and the mice were analyzed 2 days after the final PHZ injection. Mice subjected to uIRI were intraperitoneally injected with Fe-Dex (10 mg iron/mouse) 7 and 8 days after uIRI and analyzed 14 days after uIRI.[25,40] Nuclear First Red was used for counterstaining. Dotted circles indicate glomeruli. (C) Flow cytometry data showing that TfR expression in Replic cells, a myofibroblastic cell line derived from mouse REP cells,[42] was enhanced by incubation with the iron chelator deferoxamine (DFO, 100 μM) for 24 h. (D) The putative mechanism of iron incorporation into myofibroblasts in injured kidneys and the impact of iron on the myofibroblastic features related to kidney disease progression. See color figure in the on-line version.

to hypoxic inactivation. Therefore, HIF-PH inhibitors (PHD inhibitors) have been developed to block over-activated PHDs and are currently used for treatment of renal anemia.[43–46]

Further progression of myofibroblastic transformation silences *EPO* and *EPAS1* (HIF2α) gene expression by inducing DNA methylation in the promoter regions of these genes.[42,43,47] Epigenetic silencing means that HIF-PH inhibitors cannot induce *EPO* gene expression in terminally mature renal myofibroblasts due to a lack of HIF2α synthesis at the transcriptional level. Thus, studying REP cell transformation is critical for understanding renal fibrosis and renal anemia, which are major complications of CKD.

Iron Preferentially Accumulates in REP Cells and Inhibits EPO Production

Iron overload in mice fed a high-iron diet or subjected to iron-dextran injection reduced renal EPO production.[25,48] Under iron overload conditions, iron deposition is observed exclusively in renal interstitial fibroblasts, including REP cells, in the kidney (Fig. 2A and B), and hypoxia-inducible HIF2α nuclear accumulation in these cells is inhibited. Although the mechanism of iron-mediated HIF2α suppression has not been elucidated due to the complex effects of iron on HIF2α activity (Fig. 1), HIF2α inactivation results in a lack of EPO production followed by the development of renal anemia. Because iron supports erythropoiesis, iron supplementation is often provided to patients suffering from iron deficiency anemia and also to endurance athletes. However, iron overdose may attenuate erythropoietic activity by reducing HIF2α-inducible EPO production.

Intriguingly, iron deposition is detected exclusively in tubular epithelial cells after the induction of hemolytic anemia, which increases blood concentrations of heme-iron derived from the Hb of degraded erythrocytes. In contrast, renal interstitium-specific iron deposition was observed in mice administered a high-iron diet or iron dextran (Fe-Dex), and blood concentrations of Tf-iron were increased (Fig. 2A and B).[25,49] These observations suggest that REP cells incorporate Tf-iron from interstitial fluid, whereas tubular cells absorb heme-iron from the tubular lumen. Indeed, we detected *Tfrc* (TfR) mRNA expression in the REP cells of mouse kidneys. Additionally, the heme importer [heme regulatory gene 1 (HRG1), also known as SLC48A1] is expressed in the apical membrane of renal proximal tubular cells and reabsorb heme from primary urine in mice suffering from neonatal hemolysis.[50]

Roles of Iron in Myofibroblasts in Fibrotic Kidneys

Iron deposition in the interstitium is increased in kidneys damaged by ischemia–reperfusion injury (Fig. 2B), indicating that REP cells enhance Tf-iron absorption after myofibroblastic transformation under disease conditions.[41] Since EPO deficiency attenuates iron use for Hb synthesis followed by an increase in serum Tf-iron levels,[31] the incorporation of Tf-iron into renal myofibroblasts is thought to be associated with renal fibrosis and EPO deficiency in the context of CKD. Flow cytometry demonstrated that TfR expression on the cell surface of Replic cells was enhanced by a reduction in intracellular iron availability (Fig. 2C and D), iron absorption in renal myofibroblasts was thought to be controlled by regulating functional TfR expression in response to changes in cellular status.

Iron is considered to be required for proliferative myofibroblasts in fibrotic kidneys through supporting mitochondrial energy production (Fig. 2D).[51] Additionally, because excessive ferrous iron produces hydroxyl radicals in cells, iron seems to be involved in the development of oxidative stress, which is one of the major causes of tubular damage in kidney injury (Fig. 2D).[41,52] Furthermore, iron accumulation results in HIF2α inactivation and EPO deficiency in the kidney (Fig. 2D).[31] EPO deficiency leads to anemia, and anemic hypoxia further exacerbates renal fibrosis and kidney damage.[41] We recently found that iron and oxygen conditions cooperatively controlled the activity

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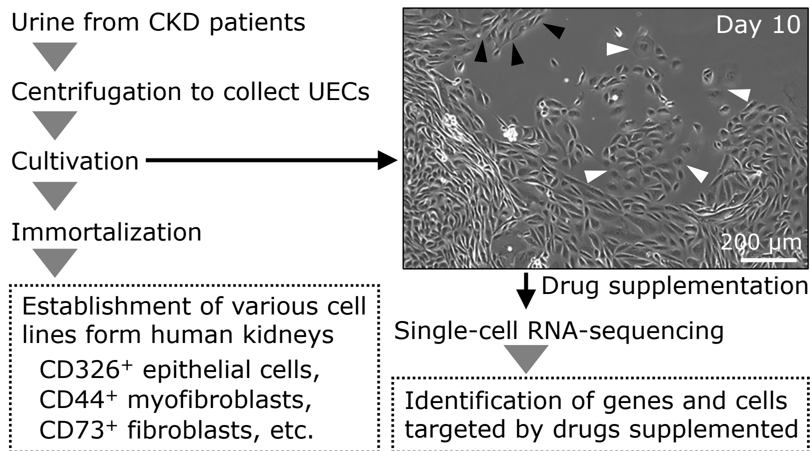

Fig. 3. The use of urinary exfoliated renal cells (UECs) of CKD patients. UECs were noninvasively isolated by centrifuging urine from CKD patients and vigorously grown under standard culture conditions. Because UECs consist of various kidney cells, including interstitial fibroblasts, myofibroblasts and tubular epithelial cells, a variety of cell lines derived from the kidneys of CKD patients can be established by cloning immortalized UECs. Additionally, UECs can be used to screen genes and cells targeted by drugs via single-cell RNA sequencing. Black and white arrowheads indicate elongated bipolar fibroblastic cells and regular polygonal epithelial cells, respectively.

of ten-eleven translocation DNA demethylases (TET),[53] which may affect the epigenetic silencing of the genes encoding HIF2α and EPO in fibrotic kidneys. These findings suggest that signaling pathways related to iron are potential molecular targets in CKD.

Perspective: Use of Urinary Exfoliated Cells from CKD Patients for Studies on EPO Production and Renal Fibrosis

Investigations using cells derived from CKD patients are essential for applying advanced information on the molecular pathology of kidney disease obtained from experiments using animal models and cells such as Replic cells. It is known that the urine of CKD patients contains a variety of cell types that are exfoliated from the injured kidneys.[43,54,55] Because urinary exfoliated cells (UECs) are living and culturable cells that are noninvasively obtained from patients, they can be used for *in vitro* experiments to investigate the molecular pathology of kidney disease, to screen biomarkers related to disease conditions and prognosis and to diagnose individual responsiveness to drugs.

Because urine concentrations of UECs are low even in CKD patients, few cells can be detected in a 10-cm dish culture immediately after seeding cells collected via the centrifugation of 40 ml of urine from CKD patients. However, cell clusters with various shapes appear within 1 week after the initiation of culture (Fig. 3), indicating high proliferation. We confirmed that cultured UECs contained $CD73^+$ fibroblasts, $CD326^+$ tubular epithelial cells, $CD44^+$ injured cells from tubules and fibroblasts, although the ratio of each cell population differed among patients. Additionally, there are cells that can produce EPO, which are human REP cells, in response to HIF-PH inhibitors.

Taking advantage of the diverse cell types of living UECs, a variety of human kidney cell lines, which are useful for elucidating the molecular pathology of CKD, can be established after the transfection of immortalizing genes, such as those encoding Simian virus 40 T-antigens (Fig. 3). Additionally, single-cell RNA sequencing of UECs exposed to drugs of interest is available for screening for the cellular and molecular targets of newly developed drugs for kidney disease (Fig. 3). For example, we are investigating the effects of iron on hypoxia- or HIF-PHI-inducible EPO production in UEC-derived fibroblastic cells that are positive for the cell-surface marker CD73. Thus, UECs are expected to noninvasively provide innovative platforms for studying the molecular pathology and pharmaceutical and diagnostic treatment of CKD, which is complicated by high unmet medical needs.

Author Contributions

NS, YI, KS, NU, NKumagai, and TN developed the project. NS drafted the manuscript. NS, YI, KK, and TN prepared the figures. YI, HI, YK, NU, NKoida, HS, NKumagai, and TN reviewed the manuscript for intellectual content. All the authors approved the publication of this paper.

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Abbreviations

CKD	chronic kidney disease
DFO	deferoxamine
EPO	erythropoietin
EPOR	erythropoietin receptor
Fe-Dex	iron dextran
Hb	hemoglobin
HIF	hypoxia-inducible factor
HIF-PH	HIF-prolyl hydroxylase (also known as PHD)
IRP	iron regulatory protein
PHD	prolyl hydroxylase domain protein
REP cell	renal erythropoietin production cell

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STAT	signal transducer and activator of transcription
TfR	transferrin receptor
UECs	urinary exfoliated cells
uIRI	unilateral ischemia–reperfusion injury

Conflict of Interest

No potential conflicts of interest were disclosed.

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