Hypoxia-inducible factor prolyl-4-hydroxylation in FOXD1 lineage cells is essential for normal kidney development

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Hanako Kobayashi[1,2], Jiao Liu[3,4], Andres A. Urrutia[1], Mikhail Burmakin[5], Ken Ishii[1], Malini Rajan[1], Olena Davidoff[1,2], Zubaida Saifudeen[3,4] and Volker H. Haase[1,2,6]

[1]Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee, USA; [2]Medical and Research Services, Department of Veterans Affairs Hospital, Tennessee Valley Healthcare System, Nashville, Tennessee, USA; [3]Section of Pediatric Nephrology, Department of Pediatrics, Tulane University Health Sciences Center, New Orleans, Louisiana, USA; [4]The Hypertension and Renal Centers of Excellence, Tulane University Health Sciences Center, New Orleans, Louisiana, USA; [5]Division of Vascular Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Stockholm, Sweden; and [6]Department of Cancer Biology and Department of Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee, USA

Hypoxia in the embryo is a frequent cause of intra-uterine growth retardation, low birth weight, and multiple organ defects. In the kidney, this can lead to low nephron endowment, predisposing to chronic kidney disease and arterial hypertension. A key component in cellular adaptation to hypoxia is the hypoxia-inducible factor pathway, which is regulated by prolyl-4-hydroxylase domain (PHD) dioxygenases PHD1, PHD2, and PHD3. In the adult kidney, PHD oxygen sensors are differentially expressed in a cell type–dependent manner and control the production of erythropoietin in interstitial cells. However, the role of interstitial cell PHDs in renal development has not been examined. Here we used a genetic approach in mice to interrogate PHD function in FOXD1-expressing stroma during nephrogenesis. We demonstrate that PHD2 and PHD3 are essential for normal kidney development as the combined inactivation of stromal PHD2 and PHD3 resulted in renal failure that was associated with reduced kidney size, decreased numbers of glomeruli, and abnormal postnatal nephron formation. In contrast, nephrogenesis was normal in animals with individual PHD inactivation. We furthermore demonstrate that the defect in nephron formation in PHD2/PHD3 double mutants required intact hypoxia-inducible factor-2 signaling and was dependent on the extent of stromal hypoxia-inducible factor activation. Thus, hypoxia-inducible factor prolyl-4-hydroxylation in renal interstitial cells is critical for normal nephron formation.

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Correspondence: *Volker H. Haase, Department of Medicine, Division of Nephrology & Hypertension, Vanderbilt University Medical Center, C-3119A MCN, 1161 21st Avenue So., Nashville, Tennessee 37232-2372, USA. E-mail: volker.haase@vanderbilt.edu*

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Hypoxia occurs not only under pathologic conditions but also physiologically during normal development and regulates stem cell behavior, cellular differentiation, proliferation, and migration, as well as the reciprocal interactions between different cell types on multiple levels, thus affecting the morphogenesis of the embryo and placenta. Therefore, molecular mechanisms that permit cells to adequately respond to discrepancies between oxygen demand and supply are critically important for normal embryonic development. A disruption of these responses may lead to developmental abnormalities in multiple organ systems and in worst case scenario to intraembryonic demise.[1]

A major and critical component of cellular hypoxia responses is the prolyl-4-hydroxylase domain (PHD)-hypoxia-inducible factor (HIF) axis, which enables cells to respond to changes in tissue oxygen levels in a rapid and controlled manner. HIF-1 and HIF-2 are pleiotropic basic helix-loop-helix transcription factors that consist of an oxygen-sensitive α-subunit and a constitutively expressed β-subunit, also known as the aryl hydrocarbon receptor nuclear translocator. HIF transcription factors regulate a multitude of hypoxia responses allowing cells to adapt to and survive low oxygen environments.[2] Under normoxic conditions, the oxygen-, iron-, and 2-oxoglutarate-dependent PHD proteins PHD1, PHD2, and PHD3, also known as egl nine homolog (EGLN)2, EGLN1, and EGLN3, respectively, function as oxygen sensors of HIF pathway. PHD enzymes initiate rapid proteasomal degradation of constitutively synthesized HIF-α subunits through the hydroxylation of specific proline residues.[3] A reduction in PHD catalytic activity, for example, under hypoxic conditions or owing to pharmacologic inhibition, results in HIF-α stabilization and activation of HIF transcriptional programs.[3]

Sustained discrepancies between oxygen demand and supply can result from maternal disease, uteroplacental insufficiency, or exposure to high altitude. This frequently leads to intrauterine growth retardation and low birth weight and increases the risk of developing diabetes, cardiopulmonary disease, stroke, arterial hypertension, or chronic kidney

disease in adults.[4–6] In the developing kidney, hypoxia reduces ureteric bud (UB) branching and nephron formation[7] and results in low nephron endowment, which by itself is associated with an increased risk of chronic kidney disease and/or arterial hypertension.[8,9]

Normal kidney development is driven by multiple reciprocal and cyclical interactions between UB and metanephric mesenchyme, which result in repeated UB branching and nephron formation.[10,11] However, little is known about the role of stromal cells in this process. The renal stroma is identified by the expression of the forkhead box D1 (FOXD1) transcription factor and surrounds the cap mesenchyme (CM). FOXD1-expressing stroma plays a critical role in renal capsule development, renal progenitor differentiation, and nephron formation; is important for normal vascular patterning; and ultimately gives rise to cortical and medullary interstitial fibroblast-like cells, pericytes, mesangial cells, and vascular smooth muscle cells.[12–17] Because hypoxia physiologically occurs during kidney development, both HIF-1α and HIF-2α have been detected in the developing kidney in a cell type–dependent manner.[18–20] However, despite clear evidence of HIF pathway activation, the functional roles of cell-specific HIF signaling during renal development are poorly understood, and information from genetic models is limited.

To examine the role of interstitial HIF oxygen sensing in renal development and homeostasis, we used the Cre-loxP system to target all 3 HIF-PHDs in conjunction with HIF-1α or HIF-2α in FOXD1-expressing stromal cells. We found that mice with individual *Phd1*, *Phd2,* or *Phd3* deletion or *Phd1-Phd2* and *Phd1-Phd3* double deletion were born with normal kidneys, whereas the combined inactivation of *Phd2* and *Phd3* resulted in abnormal kidney development, renal failure, and premature death. Kidney defects in *Phd2-Phd3* double-knockout mice became apparent after postnatal day (P) 7, correlated with the degree of interstitial HIF activation and were characterized by a HIF-2–dependent reduction in the number of mature nephrons and glomeruli as well as abnormal renal vasculature. Taken together, our data establish that the ability to regulate HIF prolyl-4-hydroxylation in FOXD1 stroma–derived cells is essential for normal nephron formation. Our data have implications for the therapeutic use of HIF prolyl-4-hydroxylase inhibitors, which are currently in phase 3 clinical development for renal anemia.[21]

RESULTS

Combined inactivation of *Phd2* and *Phd3* in FOXD1 stroma is associated with renal failure and juvenile lethality

Interstitial cells play an important role in the regulation of renal hypoxia responses. A classic example is the hypoxic induction of *erythropoietin* (EPO). To examine the role of individual PHDs in these responses, we utilized $Foxd1^{cre/+}$ transgenic mice. In this transgenic line, the *Cre* transgene, which consists of an enhanced green fluorescent protein–Cre-recombinase fusion protein, is under the transcriptional control of the *Foxd1* promoter (Figure 1a).[22,23] FOXD1-expressing cells surround the CM in the nephrogenic zone, give rise to all stromal components of the developing kidney and express *Phd1*, *Phd2,* and *Phd3* (Supplementary Figure S1).

Utilizing $Foxd1^{cre/+}$ transgenic mice, we developed mice with individual or combined inactivation of PHD1, PHD2, and PHD3. $Foxd1^{cre/+}$ $Phd1^{fl/fl}$, $Foxd1^{cre/+}$ $Phd2^{fl/fl}$, *and* $Foxd1^{cre/+}$ $Phd3^{fl/fl}$ mutant mice (hereon referred to as *Foxd1-Phd1*$^{-/-}$, *Foxd1-Phd2*$^{-/-}$, *and Foxd1-Phd3*$^{-/-}$ mutants, respectively) developed normally into adulthood, whereas $Foxd1^{cre/+}$ $Phd2^{fl/fl}$ $Phd3^{fl/fl}$ double-knockout mice (hereon referred to as *Foxd1-Phd2*$^{-/-}$*-Phd3*$^{-/-}$ mutants) were small and died prematurely (Figure 1b). Differences in whole-body weight between mutants and Cre^{-} littermate controls ($Foxd1^{+/+}$ $Phd2^{fl/fl}$ $Phd3^{fl/fl}$) became statistically significant by P14 (5.0 ± 0.4 g vs. 7.3 ± 0.5 g; n = 8 and 12, respectively; $P = 0.003$; Supplementary Table S1). Juvenile lethality in the mutant cohort was associated with renal failure and severe pathologic changes in the kidney (Figures 1c and d). Kidney weight in mutants was significantly reduced compared with that in controls (28.2 ± 2.1 mg vs. 47.9 ± 2.5 mg; n = 16 and 24, respectively; $P < 0.0001$; Supplementary Table S1), and renal defects in *Foxd1-Phd2*$^{-/-}$*-Phd3*$^{-/-}$ mutants at weaning age were characterized by tubular and vascular dilatations, tubular cyst formation, accumulation of α-smooth muscle actin (alpha actin-2 [ACTA2])–positive interstitial and glomerular cells, glomerular sclerosis, increase in collagen matrix and *collagen type 1 alpha 1* mRNA production, and increased *F4/80* mRNA levels (Figure 1e and f; Supplementary Figure S2). Because PHD inhibition results in normoxic HIF-α stabilization and HIF signaling activation, we assessed mRNA expression levels of HIF target gene *Epo*. Despite the presence of severe morphologic defects, *Epo* mRNA levels in the kidney were significantly increased in 2-week-old *Foxd1-Phd2*$^{-/-}$*-Phd3*$^{-/-}$ mice, indicating that the combined deletion of *Phd2* and *Phd3* resulted in the robust activation of the HIF system in FOXD1 stroma–derived interstitial cells (an approximate 80-fold increase; n = 9 and 10, respectively; $P <$ 0.0001; Figure 1e). Increased *Epo* was accompanied by a small but significant increase in hematocrit levels (38.8% ± 1.1% in mutants and 35.5% ± 0.8% in controls; n = 14 and 14, respectively; $P < 0.05$).

Because HIF is known to promote angiogenesis,[24,25] we examined the renal vasculature in *Foxd1-Phd2*$^{-/-}$*-Phd3*$^{-/-}$ kidneys. For this, we used real-time polymerase chain reaction (PCR) in conjunction with immunohistochemistry to characterize the tissue expression patterns of ACTA2 and endothelial cell marker cluster of differentiation (CD)31. On P14, we observed significant increases in microvessel density, which correlated with elevated *Cd31* mRNA expression levels in whole-kidney homogenates (approximately 1.4-fold increase; Figure 1f). Furthermore, small- and medium-sized arterial vessels in *Foxd1-Phd2*$^{-/-}$*-Phd3*$^{-/-}$ kidneys were dilated and characterized by relatively thin vessel walls (Figure 1d). Vascular changes in *Foxd1-Phd2*$^{-/-}$*-Phd3*$^{-/-}$ kidneys were associated with increased transcription of *vascular endothelial growth factor* in the renal interstitium, as