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Activation of Hypoxia Signaling in Stromal Progenitors Impairs Kidney Development

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Intrauterine hypoxia is a reason for impaired kidney development. The cellular and molecular pathways along which hypoxia exerts effects on nephrogenesis are not well understood. They are likely triggered by hypoxia-inducible transcription factors (HIFs), and their effects appear to be dependent on the cell compartment contributing to kidney formation. In this study, we investigated the effects of HIF activation in the developing renal stroma, which also essentially modulates nephron development from the metanephric mesenchyme. HIF activation was achieved by conditional deletion of the von Hippel–Lindau tumor suppressor (VHL) protein in the forkhead box FOXD1 cell lineage, from which stromal progenitors arise. The resulting kidneys showed maturation defects associated with early postnatal death. In particular, nephron formation, tubular maturation, and the differentiation of smooth muscle, renin, and mesangial cells were impaired. Erythropoietin expression was strongly enhanced. Codeletion of *VHL* together with *HIF2A* but not with *HIF1A* led to apparently normal kidneys, and the animals reached normal age but were anemic because of low erythropoietin levels. Stromal deletion of *HIF2A* or *HIF1A* alone did not affect kidney development. These findings emphasize the relevance of sufficient intrauterine oxygenation for normal renal stroma differentiation, suggesting that chronic activity of HIF2 in stromal progenitors impairs kidney development. Finally, these data confirm the concept that normal stroma function is essential for normal tubular differentiation. *(Am J Pathol 2017, ■: 1–16; http://dx.doi.org/10.1016/j.ajpath.2017.03.014)*

Q4 Although mammalian kidneys normally develop in a hypoxic state, a further decrease of intrauterine oxygen availability, such as in states of placental insufficiency, impairs nephrogenesis and in consequence kidney development, causing life-long health risks.[1–5] The cellular and molecular mechanisms along which intrauterine hypoxia affects kidney development appear to be complex, and they are yet poorly understood. There appears to exist an optimal low range of tissue oxygen concentrations outside of which kidney development is impaired. Moreover, hypoxia of different cell compartments contributing to kidney formation may exert differential effects on kidney differentiation. The metanephric kidney develops from three major cell pools (namely, ureteric buds, metanephric mesenchyme, and stromal progenitors).[6] These different cell precursor pools are characterized by their dependency on specific transcription factors. Homeobox protein HOXB7-dependent precursor cells produce ureteric buds and collecting ducts. Homeobox protein SIX2-dependent precursor cells lead to all epithelial cells of the nephron, including podocytes. Finally, forkhead box FOXD1 dependency defines stromal progenitors developing to interstitial fibroblasts and pericyte-like cells, vascular smooth muscle, mesangial cells, and renin-producing cells.[6–8]

It is commonly assumed that the effects of hypoxia on differentiation are transmitted by hypoxia-inducible transcription factors (HIFs). It has been shown recently that HIF activation in the ureteric buds favors nephrogenesis, whereas HIF activation in the metanephric mesenchyme seems to impair nephrogenesis.[9] To achieve a better understanding of the mechanisms along which hypoxia affects kidney development, it appears therefore reasonable

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to first analyze hypoxia-induced events in defined cell compartments contributing to the developing kidney. Activation of HIFs in the SIX2 cell lineage producing all epithelial cells of the nephron led to viable mice that developed cysts and fibrosis after several months of life.[10] Another study in which HIF1 was activated in the HOXB7 cell lineage producing ureteric buds, collecting ducts, and parts of the distal nephron reported the development of viable mice, which developed progressive fibrosis and dilatory tubular changes at an age of 2 months.[11] In line, stable expression of HIF1 in tubular cells *in vivo* promotes renal fibrosis.[12] Although the stromal cell compartment defined by dependency on FOXD1 also essentially modulates nephron development from the metanephric mesenchyme,[13,14] the effects of hypoxia in stromal precursors on kidney development have so far not been investigated. We therefore analyzed the effects and functional consequences of HIF activation in stromal precursors on kidney development and differentiation. A common way to activate HIFs apart by hypoxia is their stabilization by inhibition/deletion of individual prolyl 3-hydroxylases (P3Hs)[15–21] or by deletion of the von Hippel–Lindau tumor suppressor (*VHL*) gene, which is centrally involved in proteasomal HIF degradation.[22,23] Because more than one P3H isoform appears to be relevant for HIF regulation in the renal FOXD1 cell compartment,[15] we chose deletion of *VHL* from the FOXD1 compartment as the maneuver for general HIF stabilization. For this purpose, we have generated and characterized a mouse strain carrying *Cre* recombinase under the control of the *FOXD1* promoter and carrying two floxed *VHL* alleles ($FOXD1^{+/Cre}$ $VHL^{fl/fl}$ mice). To confirm HIF dependency of effects observed, we further generated and characterized mice lacking *HIF1A* or *HIF2A* in the FOXD1 cell lineage.

We found that deletion of *VHL* strongly attenuated the differentiation of cells directly deriving from the FOXD1 department. In addition, nephrogenesis was delayed, leading to insufficient kidney function being the likely reason for the early postnatal death. All these changes induced by *VHL* deletion from the FOXD1-positive compartment were dependent on HIF2 but not on HIF1.

Materials and Methods

Study Approval

Institutional review boards for the NIH and the University of Regensburg reviewed and approved the present study, which was performed under the guidelines of NIH's *Guide for the Care and Use of Laboratory Animals*.[24]

Animals

Mice with a conditional deletion of *VHL* in FOXD1-expressing cells ($FOXD1^{+/Cre}$ $VHL^{fl/fl}$ mice) were generated by crossbreeding mice with loxP flanked *VHL* alleles[25] and mice with a $FOXD1^{tm1(GFP/cre)Amc}$ allele, which expresses an enhanced green fluorescent protein (eGFP)–Cre fusion protein from the *FOXD1* promoter/enhancer elements[7,26] (referred to as $FOXD1^{+/Cre}$ mice: 012463; purchased from Jackson Laboratories, Bar Harbor, ME). FOXD1 cell-specific knockout mice for *VHL* and *HIF2A* ($FOXD1^{+/Cre}$ $VHL^{fl/fl}$ $HIF2A^{fl/fl}$) or only for *HIF2A* ($FOXD1^{+/Cre}$ $HIF2A^{fl/fl}$) were generated by crossing the previously mentioned mouse strains with mice with loxP flanked *HIF2A* alleles.[27] FOXD1 cell-specific knockout mice for *VHL* and *HIF1A* ($FOXD1^{+/Cre}$ $VHL^{fl/fl}$ $HIF1A^{fl/fl}$) or only for *HIF1A* ($FOXD1^{+/Cre}$ $HIF1A^{fl/fl}$) were generated by crossing the previously mentioned mouse strains with mice with loxP flanked *HIF1A* alleles.[28] $FOXD1^{+/Cre}$ littermates served as controls. For tracing of the FOXD1 cell lineage, $FOXD1^{+/Cre}$ mice were crossed with the membrane-Tomato/membrane-GFP (mT/mG) dual fluorescent reporter mouse strain [007676; purchased from Jackson Laboratories; the *mT/mG* reporter expresses membrane-targeted tdTomato (mT); when bred to Cre recombinase–expressing mice, the resulting offspring have the *mT* cassette deleted in the Cre-expressing tissue(s), allowing expression of the membrane-targeted *eGFP* (*mG*) cassette located just downstream].[29] Animals were maintained on standard rodent chow (0.6% NaCl; Ssniff, Soest, Germany) with free access to tap water. Animals used in this study were in the embryonic state of embryonic day 16/18, newborn, 7 days old, or 7 weeks old. All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals, published by the NIH, and approved by the local ethics committee.

Determination of Hematocrit, Plasma EPO, and Plasma REN Levels

Blood samples were taken into capillary tubes containing 1 μL 125 mmol/L EDTA to prevent clotting. Hematocrit values were determined after centrifugation (12,000 × *g* for 4 minutes at room temperature). The erythropoietin (EPO) protein concentration was determined in plasma samples using the Quantikine Mouse EPO ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's protocol. Renin (REN) concentration in plasma samples was measured on the basis of the generation of angiotensin-I after the addition of plasma from bilaterally nephrectomized male rats as excess renin substrate. The generated angiotensin-I (ng angiotensin-I/hour per mL) was determined by radioimmunoassay (Byk & DiaSorin Diagnostics, Dietzenbach, Germany).[30]

Determination of mRNA Expression by Real-Time PCR

Total RNA was isolated from kidneys, as described by Chomczynski and Sacchi,[31] and quantified by a

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