Hypoxia inhibits nephrogenesis through paracrine Vegfa despite the ability to enhance tubulogenesis

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Reduced nephron number predisposes to hypertension and kidney disease. Interaction of the branching ureteric bud and surrounding mesenchymal cells determines nephron number. Since oxygen supply may be critical for intrauterine development, we tested whether hypoxia and hypoxia-inducible factor-1α (HIF-1α) influence nephrogenesis. We found that HIF-1α is required for branching of MDCK cells. In addition, culture of metanephric mouse kidneys with ureteric bud cell-specific stabilization or knockout of HIF-1α revealed a positive impact of HIF-1α on nephrogenesis. In contrast, widespread stabilization of HIF-1α in metanephric kidneys through hypoxia or HIF stabilizers impaired nephrogenesis, and pharmacological HIF inhibition enhanced nephrogenesis. Several lines of evidence suggest an inhibitory effect through the hypoxia response of mesenchymal cells. HIF-1α was expressed in mesenchymal cells during nephrogenesis. Expression of the anti-branching factors Bmp4 and Vegfa, secreted by mesenchymal cells, was increased upon HIF stabilization. The conditioned medium from hypoxic metanephric kidneys inhibited MDCK branching, which was partially rescued by Vegfa antibodies. Thus, the effect of HIF-1α on nephrogenesis appears context dependent. While HIF-1α in the ureteric bud is of importance for proper branching morphogenesis, the net effect of hypoxia-induced HIF activation in the embryonic kidney appears to be mesenchymal cell-dependent inhibition of ureter branching.

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There is compelling evidence that the number of nephrons in humans correlates inversely with the risk to develop arterial hypertension or chronic kidney disease during lifetime.[1,2] However, factors influencing nephron formation remain incompletely understood. The development of the definite kidney begins when the nephric duct gives rise to the ureteric bud (UB), which subsequently invades the surrounding metanephric mesenchyme.[3] Bidirectional inductive interactions between the ductal epithelium and the nephrogenic mesenchyme result in the spatial organization of the kidney. In mice, outgrowth of the UB starts at embryonic day 10.5 (E10.5) and at day E11.5 the metanephric mesenchyme induces the UB to grow and branch, thereby giving rise to the collecting duct system.[4] In turn, the UB induces the metanephric mesenchyme to condense and differentiate into tubular structures, which will later form the nephrons.[5] The complexity of this interaction might be one of the reasons why urinary tract malformations are among the most common birth defects in humans.[6]

During development, kidneys are exposed to hypoxia.[7] One of the main cellular responses upon hypoxia is the stabilization of hypoxia-inducible transcription factors (HIF).[8] HIF consists of a heterodimer with a constitutive β-subunit (HIF-β) and an oxygen-regulated α-subunit (HIF-1α or HIF-2α).[9] In normoxia, oxygen-dependent hydroxylation of HIF-α by HIF-prolyl hydroxylases (PHDs) targets the α-subunits for proteasomal degradation.[9] Whereas both HIF-1α and HIF-2α are barely detectable in healthy adult kidneys,[10] we and others have shown that during kidney development, HIF-1α is significantly expressed in medullary and cortical collecting ducts.[11–13] The functional consequence of HIF-1α induction during embryogenesis remains unclear. Low oxygen levels have been shown to increase[14–16] or decrease UB branching of *ex vivo* cultured metanephric kidneys.[15,16] In a model of intrauterine growth restriction, nephron number was significantly reduced in the presence of increased levels of HIF-1α,[17] but whether this association between HIF-1α expression and impaired nephrogenesis is causal cannot be inferred from these findings.

To further investigate the functional role of hypoxia and HIF-1α for kidney development, we combined models of

in vitro branching and *ex vivo* cultured metanephric kidneys under conditions of varying oxygen availability and using both pharmacological and genetic approaches to modulate the HIF system *in vivo*.

RESULTS

HIF-1α is required for *in vitro* branching morphogenesis of MDCK cells

To analyze the effects of HIF-1α on branching morphogenesis, we used the well-established Madin-Darby canine kidney (MDCK) tubulogenesis model.[18] MDCK cells originate from the collecting duct and hence the UB[19] and proliferate within a collagen 1 matrix forming small cysts.[20] In the presence of hepatocyte growth factor (HGF), some of the cyst cells develop extensions (step 1) which then guide cells migrating out of the cyst wall to form a chain of cells (step 2). Cells then further divide to form a cord (step 3) and finally a lumen, resulting in tubule formation *in vitro* (step 4).[21] This process resembles the branching morphogenesis of an immortalized UB cell line.[19] Recently, we have shown that MDCK cells within this collagen matrix experience hypoxia corresponding to 3–5% O_2 under normal cell culture conditions resulting in a basal expression of HIF-1α.[22] Further stabilization of HIF-1α by 1% O_2 in the atmosphere or the PHD inhibitor dimethyloxalylglycine (DMOG)[9] significantly increased the number of extensions (Figure 1a and b). In addition, 1% O_2 and DMOG induced longer extensions and faster tubule formation, resulting in significantly elevated numbers of *in vitro* tubules (Supplementary Figure S1A–C online). In contrast, inhibition of HIF-1α by 17-N-Allylamino-17-demethoxygeldanamycin (Tanespimycin), an inhibitor of heat-shock protein 90 that promotes destabilization of HIF-1α,[23] markedly reduced extension numbers (Figure 1c). Chetomin, a more specific inhibitor of HIF-1α,[24] also inhibited *in vitro* branching morphogenesis (Figure 1d). To further confirm these findings, we generated MDCK cell clones stably expressing small hairpin RNA directed against HIF-1α, which significantly reduced its expression (Figure 1f). In line with the previous data, knockdown of HIF-1α inhibited *in vitro* branching morphogenesis (Figure 1g, Supplementary Figure S1D online).

Hypoxia and PHD inhibition impair branching morphogenesis of ex vivo cultured metanephric kidneys, whereas inhibition of HIF-1α augments *ex vivo* nephrogenesis

To test for HIF-1α-dependent effects on kidney development in the more complex organ context, we used metanephric mouse kidneys that were harvested at two different time points, E12.0–12.5 and E13.0–E13.5, representing an early phase, and a more advanced stage of UB branching.[25] Kidneys were cultured *ex vivo* for 24 h, followed by 48 h of treatment of one of the kidneys, using the contralateral kidney as control. Thereafter, kidney size, number of glomeruli, and number of branches were analyzed. Branching morphogenesis is frequently assessed by counting the UB tips, which can be precisely performed in kidneys harvested at the early time point.[14] However, the more complex branching architecture at the later time point makes reliable measurements of morphogenesis difficult. Tomographic methods that can be used for quantification of rodent kidney development in three dimensions are unsuitable to analyze high sample numbers.[26] Therefore, we established a novel microscopic technique that allowed us to reliably determine several parameters of branching morphogenesis, but was still applicable to a large number of kidneys. First, we stained whole mount metanephric kidneys with fluorescein isothiocyanate-conjugated dolichos biflorus agglutinin to detect UB branches. Next, kidneys were photographed along the z-axis to generate a set of 20 photos on average. These photos were then combined into a single extended depth of field image covering the whole branching architecture and reflecting each branch in a focused way, independent of its localization within the z-section (Figure 2a). The resulting image was further processed (Figure 2b) providing a skeleton of the branching architecture (Figure 2c and d) which was subsequently analyzed for the total number of branches. In addition, kidneys were stained for Wilms tumor 1 protein as a marker for glomeruli (Figure 2f) and images were rendered to obtain three-dimensional illustrations of the branches (Figure 2e and h) and glomeruli (Figure 2g and h). To validate this new technique, kidneys at different stages of development were analyzed, and the results were compared with manual counts obtained by three independent investigators blinded for the experimental condition (Supplementary Table S1 online), which revealed a high level of congruency. Furthermore, the reliability of our method was confirmed by close correlations of branch numbers with glomerular numbers and kidney size (Figure 2i–k).

In contrast to our observations in MDCK cells, 1% O_2 significantly reduced the size of metanephric kidneys, as well as the number of branches and glomeruli at both time points of development (Figure 3a and f). In the presence of 5% O_2, kidney size, number of branches and glomeruli were also significantly reduced at early and later stages of kidney development (Figure 3b and g), but the effect was not as pronounced as under 1% O_2. We then tested the effects of the PHD inhibitors DMOG and 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetate (ICA),[27,28] and again found markedly decreased kidney sizes, and reduced numbers of branches and glomeruli at both time points of development (Figure 3c, d, h, and i). In contrast, the HIF-1α inhibitor chetomin led to an increased number of glomeruli at the early stage (Figure 3e) and an increased number of glomeruli and branches at the later stage (Figure 3j). Taken together, these findings suggested a complex role of HIF-1α with a net inhibitory function in metanephric kidneys overcoming the HIF-1α-dependent pro-branching morphogenesis of UB cells.