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Expression of hypoxia-inducible transcription factors in developing human and rat kidneys

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Early kidney development is associated with the coordinated branching of the renal tubular and vascular system and hypoxia has been proposed to be a major regulatory factor in this process. Under low oxygen levels, the hypoxia-inducible transcription factor (HIF) regulates the expression of genes involved in angiogenesis, erythropoiesis and glycolysis. To investigate the role of HIF in kidney development, we analyzed the temporal and spatial expression of the oxygen regulated HIF-1α and -2α subunits at different stages of rat and human kidney development. Using double-staining procedures, localization of the HIF target geneproducts vascular endothelial growth factor (VEGF) and endoglin was studied in relation to HIFα. In both species, we found marked nuclear expression of HIF-1α in medullary and cortical collecting ducts and in glomerular cells. In contrast, HIF-2α was expressed in interstitial and peritubular cells podocytes of the more mature glomeruli. After completion of glomerulogenesis and nephrogenesis, HIF-1α and -2α were no longer detectable. The HIF-target gene VEGF colocalized with HIF-1α protein in glomeruli and medullary collecting ducts. HIF-2α colocalized with the endothelium-associated angiogenic factor, endoglin. Both HIFα isoforms are activated in the developing kidney in a cell-specific and temporally controlled manner, indicating a regulatory role of oxygen tension in nephrogenesis. HIF-1α seems to be primarily involved in tubulogenesis and HIF-2α in renal vasculogenesis. Both isoforms are found in glomerulogenesis, potentially having synergistic effects.

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The process of nephrogenesis is coordinated by complex molecular and physiological mechanisms that are still largely unknown. The development of the mammalian kidneys starts at days E11/12 in mice and rats and at gestational week 4–5 in humans (Figure 1). The process of nephrogenesis is initiated by the ureteric bud, which branches dichotomously and forms the origin of the collecting duct system. At the tip of the ureteric buds, mesenchymal cells form aggregates that epithelialize and initiate glomerulogenesis in four consecutive stages: (1) the vesicle stage, (2) the comma stage, (3) the S-shaped stage and (4) the definitive glomerulus.[1] The S-shaped bodies fuse with the collecting duct to form the nephron. Completion of kidney development in mice and rats takes place at days 7–8 post partum (Figure 1, upper panel). In humans, kidney development is terminated at gestational weeks 32–36 (Figure 1, lower panel).

Nephrogenesis is accompanied by the growth and development of the renal vascular system. Because of a mismatch of oxygen demand and vascularization, local oxygen tension is presumably low in early developmental stages. Thus, regional hypoxia is believed to play a major regulatory role in tissue maturation, albeit the underlying molecular mechanisms are incompletely understood. In recent years, the *hypoxia-inducible factor* (*HIF*) has been identified as a transcription factor, which is in a key position to control gene expression under low oxygen tensions.[2–4] Regulation involves angiogenesis, erythropoiesis, glycolysis, vascular tone, pH homeostasis and cell survival decisions like proliferation and apoptosis.[2,3,5] HIF is a heterodimer consisting of an oxygen regulated α-subunit (HIF-1α or HIF-2α) and a constitutively expressed β-subunit (HIFβ, aryl hydrocarbon receptor nuclear translocator). In the presence of oxygen, two critical prolines of the HIFα chains are hydroxylated by prolyl hydroxylases, which require molecular oxygen as substrate. The hydroxylated prolyl residues are recognized by the von Hippel–Lindau protein as a component of an E3 ubiquitin ligase, which targets HIFα for proteasomal degradation. Under hypoxic conditions, HIF accumulates in the cell and induces transcription of target

genes.[3,4] Studies on HIFα and HIFβ knockout mice[6–9] highlighted the importance of HIF in embryonic development. Knockout of HIF-1α resulted in severe cardiovascular malformations, defects of the neural tube and embryonic lethality at day E11.[8] Studies on HIF-2α knockout mice yielded inconsistent results in terms of lethality and phenotype. Peng *et al.*[6] described severe vascular malformations and embryonic death at day E9.5,[6] whereas Tian *et al.*[10] reported an increased but incomplete embryonic lethality at

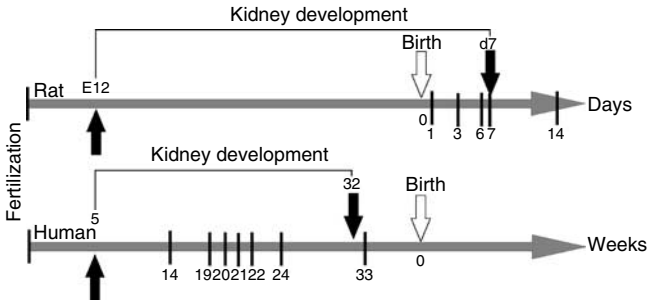

Figure 1 | Differential time course of rat and human kidney development. Renal development is initiated in early pregnancy as indicated by the black arrows (↑). Human kidney development ends at the gestional week 32 (lower panel), whereas rat renal development continues post partum (| – examined time points, ↓ – completion of kidney development, (⇩) – birth).

Table 1 | Gestational week and fetal diseases of the examined human tissue

Week of gestation	Disease of the fetus
14	Uncharacterized chromosomal aberration
19	Trisomy 21
21	Complex malformations
22	Spina bifida
24	Bursted amnion
33	Dandy–Walker syndrome

Table 2 | Expression sites of HIF-1α and HIF-2α in developing rat and human kidneys compared to their target genes endoglin and VEGF

	HIF-1α	HIF-2α	VEGF	Endoglin
Human	CD medulla	Podocytes	CD medulla	Endothelial cells
	CD cortex S-shaped bodies Glomerular cells	Endothelial cells Interstitial cells (cortex, medulla)		
Rat	CD medulla	Podocytes	CD medulla	Endothelial cells
	CD cortex Glomerular cells	Endothelial cells Interstitial cells (medulla)		

CD, collecting ducts.

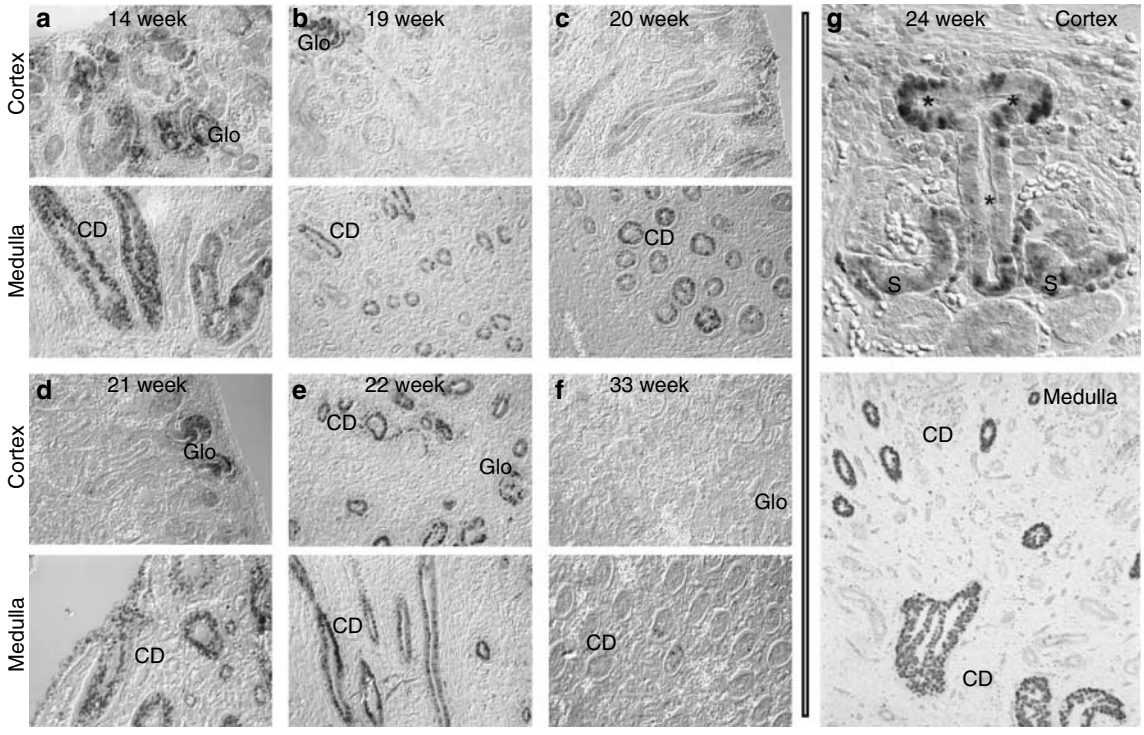

Figure 2 | HIF-1α expression in human kidney development. At the examined time points, HIF-1α was detectable from week 14 to 24 of human kidney development (**a-e**, **g**). After completion of renal development at week 33, signals for HIF-1α were almost absent (**f**). HIF-1α was strongly expressed in the nephrogenic zone in earlier stages of glomerular development (comma (**a**, **b**; cortex) and S-shaped bodies (**c-e, g**; cortex), whereas in the more mature glomeruli HIF-1α stained negative (**f**; cortex). In addition, medullary collecting ducts showed clear nuclear staining at every developmental stage (**a-e**, **g**; medulla). After completion of renal development, the collecting ducts were negative for HIF-1α (**f**, medulla). (**g**) Dividing collecting duct ampullae (*) with clear nuclear staining of HIF-1α. In direct vicinity epithelial cells of S-shapes (S) express HIF-1α as well (**g**). (CD – collecting ducts; Glo – glomerulus; Original magnification ×400; **g** cortex ×1000).

day E16.5 due to impaired catecholamine synthesis and bradycardia. Scortegagna *et al.*[11] described normal vasculogenesis and viability in HIF-2$\alpha^{-/-}$ mice, but a shortened lifespan due to complex metabolic changes, myopathy, cardiac hypertrophy and pancytopenia. To date, the functional role of HIF in renal development is still unknown, partially, because of early embryonic lethality of the knockouts at a developmental stage when kidney development is just initiated.

In mouse and human fetus, the mRNA of HIF-1α and -2α is present at different stages of kidney development.[12,13] However, HIF is regulated predominantly at the post-translational level by hydroxylation and proteasomal degradation.[14] Therefore, cellular expression of HIF proteins has to be validated on the protein level. In previous studies, HIF protein was either not detectable[15] or was restricted to the distal convoluted tubules[16] under normoxic conditions in the adult rodent kidney. Irrespective of differences in study design or applied methods, HIF was strongly inducible in any case by low oxygen tensions in the adult kidney.[15,16] In a recent study, Freeburg and Abrahamson[12] demonstrated that HIF-2α protein is expressed particularly in podocytes of the glomeruli. Immunoprecipitation and Western blot analysis indicated the presence of HIF-1α/HIFβ dimers in lysates of newborn mouse kidneys, but the authors did not demonstrate HIF-1α protein on a cellular level.

Based on these reports of HIF-mRNA and -2α protein expression in the *mouse*, we looked for the temporal and spatial expression of HIF-1α in specimens of *human* nephrogenesis and in the *rat* as a model organism, and investigated how HIF-1α is related to HIF-2α protein expression on a cellular level. Furthermore, we examined how two important HIF target genes, vascular endothelial growth factor (VEGF) and endoglin, which are involved in renal angiogenesis, are related to the expression of the HIF isoforms.

RESULTS

Expression of HIF-1α and HIF-2α in human kidney development

Because nephrogenesis is completed in humans at gestational weeks 32–36, we investigated kidneys of human aborts with different underlying diseases between weeks 14 and 33 (Table 1). HIF-1α protein was detectable in the medulla and most pronounced in the nephrogenic zone of the examined kidneys (Table 2, Figure 2a–e, g), except at gestational week 33 (Figure 2f). In particular, strong signals for HIF-1α were detected in the nuclei of epithelial cells in sprouting uretric buds of the medulla and cortex (Figure 2g). The nephrogenic zone showed marked nuclear staining of HIF-1α in epithelial cells of branching ampullae and in cells of S-shaped bodies, where the proximal parts of the nephron originate (Figure 2g).

For direct comparison, we used sections of the same specimens to analyze HIF-2α distribution. In contrast to the marked staining of HIF-1α in the outer cortex, HIF-2α staining was more pronounced in cortical and medullary interstitial cells (Figure 3a) and in podocytes of the more mature glomeruli of the inner cortex (Figure 3b). Furthermore, endothelial cells showed only HIF-2α (Figure 3c) but no HIF-1α expression. In order to test for correlations of HIFα isoforms and cellular proliferation, we performed immunohistochemistry for the proliferation marker KI67. KI67 was predominantly found in the nephrogenic zone, either in tubular cells of sprouting collecting ducts or in glomerular and endothelial cells, overall showing no clear correlation to HIF-1α or -2α (data not shown). A kidney in the 33rd week of gestation, when tubulogenesis and glomerulogenesis had come to an end, did not show any HIF-2α expression (data not shown). Even though these findings are based on the examination of single samples from fetuses with multiple abnormalities, the cellular distribution pattern of HIFα isoforms within the human kidneys investigated was very consistent. The distribution of HIFα in developing human kidneys is summarized in Table 2.

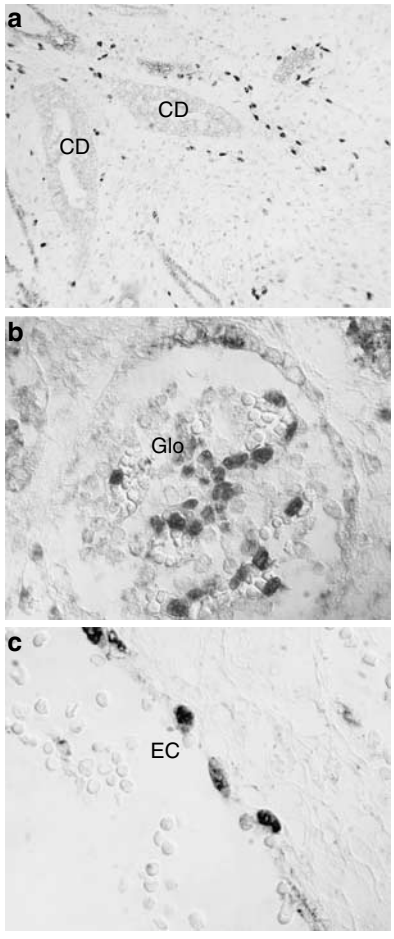

Figure 3 | HIF-2α expression in human kidney development. The kidney of the 24th gestational week shows strong accumulation of HIF-2α in cortical (data not shown) and medullary interstitial cells (**a**, medulla, original magnification × 400) and in podocytes of the more mature, juxtamedullary glomeruli (**b**, original magnification × 1000). In addition, marked induction of HIF-2α was evident in endothelial cells (**c**, original magnification × 1000). (CD – collecting ducts; EC – endothelial cells; Glo – glomerulus).

Expression of HIF-1α and HIF-2α in rat kidney development

Nephrogenesis is completed in mice and rats at day 7 post partum. In comparison to human kidney samples, we investigated rat kidneys at days 1, 3, 6, 7 and 14 post partum for HIF-1α and -2α protein expression by immunohistochemistry. Marked signals for HIF-1α were found predominantly in the medulla (Figure 4a–c, lower panels) and only rarely in the cortex (Figure 4a–c, upper panels). In detail, until 7 days post partum, medullary collecting duct cells stained positive for HIF-1α (Figure 5a and c), whereas in the nephrogenic zone HIF-1α protein was only sporadically detectable in glomerular cells (Figure 5b). In contrast, HIF-2α was only expressed in podocytes at the first day post partum (Figure 4d, upper row) and was no longer detectable in cells of the renal cortex at 6 and 14 days post partum, respectively (Figure 4e and f, upper row). In interstitial cells of the inner medulla, HIF-2α was strongly expressed at early postnatal stages (Figure 4d and e, lower row) but disappeared until day 14 when kidney development was completed (Figure 4f, lower row). The distribution of HIFα in the postnatal rat kidney is summarized in Table 2.

Expression of HIF-1α and -2α in developing rat kidneys in response to hypoxia

To test for modulation of HIF expression in the developing kidney by systemic hypoxia, we exposed newborn rats at the age of 3 days to 8% oxygen for 4.5 h. Compared to normoxic controls (Figure 6a and c), hypoxia led to a further increase of HIF-1α (Figure 6b) and HIF-2α (Figure 6d) protein signals, without change in cellular distribution. In hypoxia, virtually all nuclei of papillary collecting duct cells stained positive for HIF-1α (Figure 6b). In comparison, HIF-2α was predominantly positive in interstitial cells (Figure 6d). Occurrence and tissue distribution of hypoxia-induced HIF-1 and -2α proteins were similar to HIF staining patterns observed in hypoxic kidneys of adult rats.

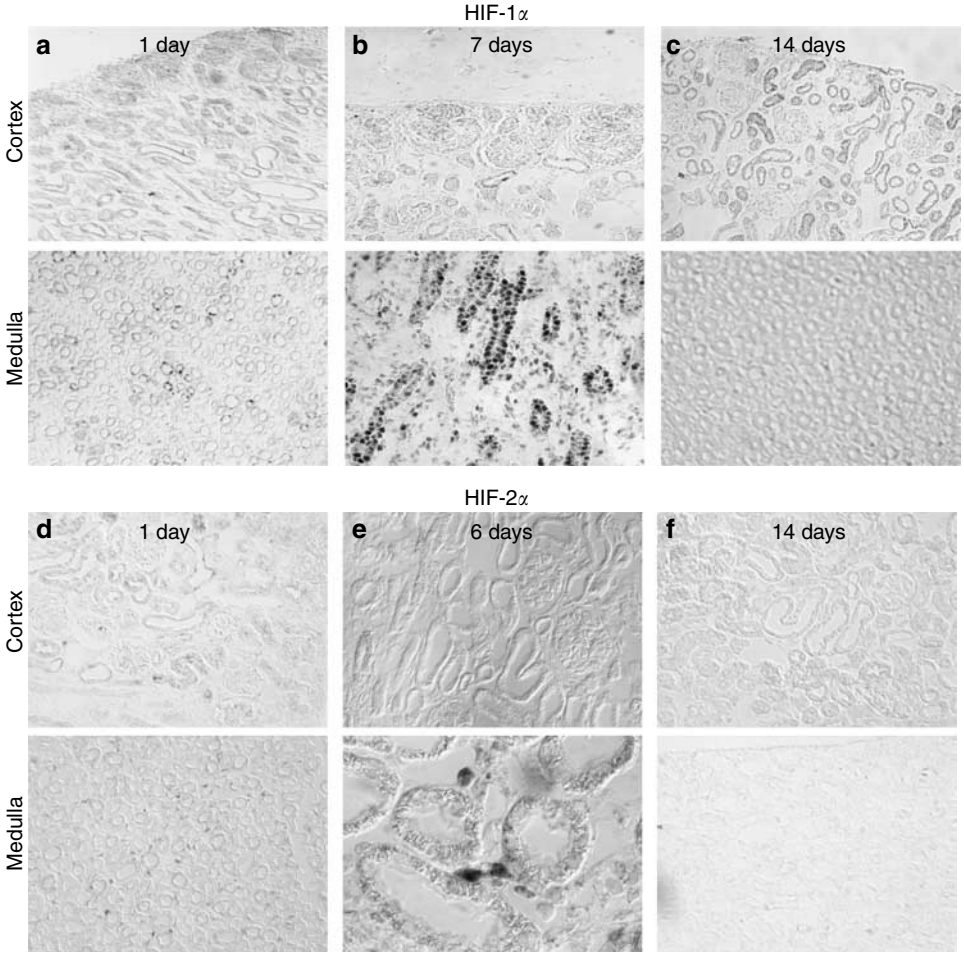

Figure 4 | HIFα expression in rat kidney development. Both HIFα isoforms were detectable up to 6–7 days post partum (**a**, **b**, **d**, **e**). In contrast, 14 days after birth, in the rat kidneys, no HIF signals were detectable any more (**c**, **f**). Up to 7 days post partum, the renal cortex exhibits only sporadic staining for HIF-1α (**a**, **b**, upper panels, original magnification × 400), but in medullary collecting ducts, HIF-1α accumulates extensively (**a**, **b**, lower panels, original magnification × 400). One day after birth, the renal cortex exhibits only sporadic HIF-2α positive endothelial cells and sporadically in podocytes (**d**, upper panel, original magnification × 400). No expression was seen after 6 and 14 days (**e**, **f**, upper panels, original magnification × 400). In comparison, HIF-2α is expressed in interstitial cells of rat papilla upto 6 days post partum (**d**, original magnification × 400, **e**, original magnification × 2000; lower panels).

VEGF and endoglin proteins colocalize with HIF-1α and HIF-2α, respectively

To test whether target gene expression correlates with HIF isoform distribution, we performed double staining immunohistochemistry in developing human kidneys. VEGF colocalized with HIF-1α whereas endoglin colocalized with HIF-2α protein. In detail, parallel staining of VEGF and HIF-1α was found in the glomerular epithelial cells (Figure 7a) and cells of the medullary collecting duct. HIF-1α showed strong nuclear and VEGF cytoplasmic signals (Figure 7b). Cortical collecting ducts were positive for HIF-1α only (Figure 7a).

For comparison, endoglin, which is strongly expressed by endothelial cells of newly formed vessels (Figure 8a), showed no colocalization with HIF-1α (Figure 8b), but with HIF-2α positive endothelial cells (Figure 8c).

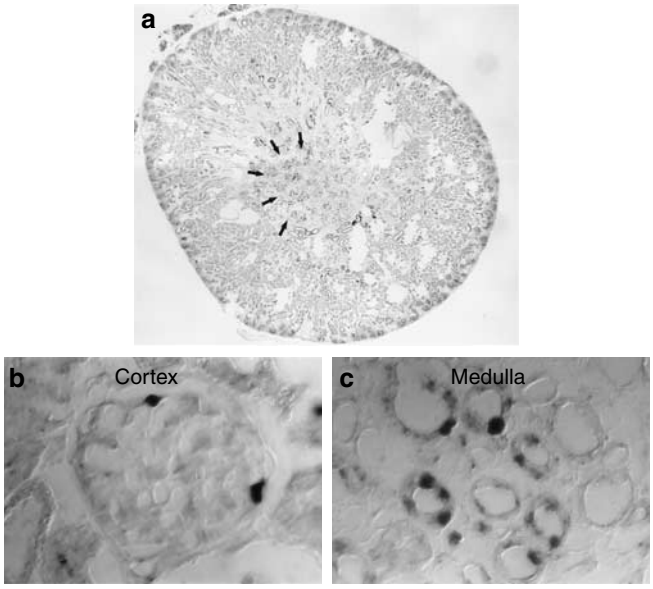

Figure 5 | HIF-1α expression in rat kidney development, 1st day post partum. (**a**) An overview of HIF-1α staining of a newborn rat kidney with predominant HIF-1α expression in the inner medulla (black arrows, **a**, original magnification × 100). Only sporadic staining of HIF-1α in the glomerular cells of the nephrogenic zone was detected (**b**, original magnification × 1000), whereas medullary collecting ducts show clear nuclear staining for HIF-1α at this age (**c**, original magnification × 1000).

DISCUSSION

This study demonstrates that HIF-1α and -2α are expressed during rat and human kidney development in a spatially and

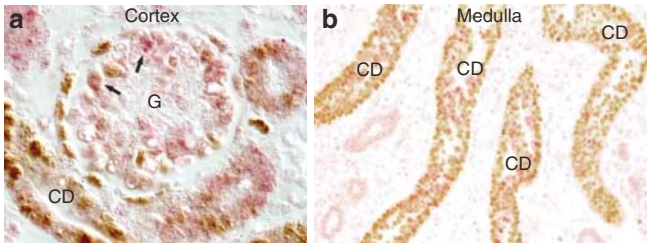

Figure 7 | Localization of the HIF target gene VEGF in human kidney development, 22nd gestational week. HIF-1α (brown, peroxidase staining) is colocalized with the HIF target gene VEGF (red, fast red staining) in human kidney development. In glomerular cells, HIF-1α and VEGF are partially coexpressed (**a**, black arrows, original magnification × 1000), whereas cortical collecting ducts stained positive for HIF-1α but not for VEGF (**a**, CD). Medullary collecting ducts showing nuclear staining for HIF-1α and cytoplasmic reactivity for VEGF (**b**, original magnification × 1000). (CD – collecting ducts; Glo – glomerulus).

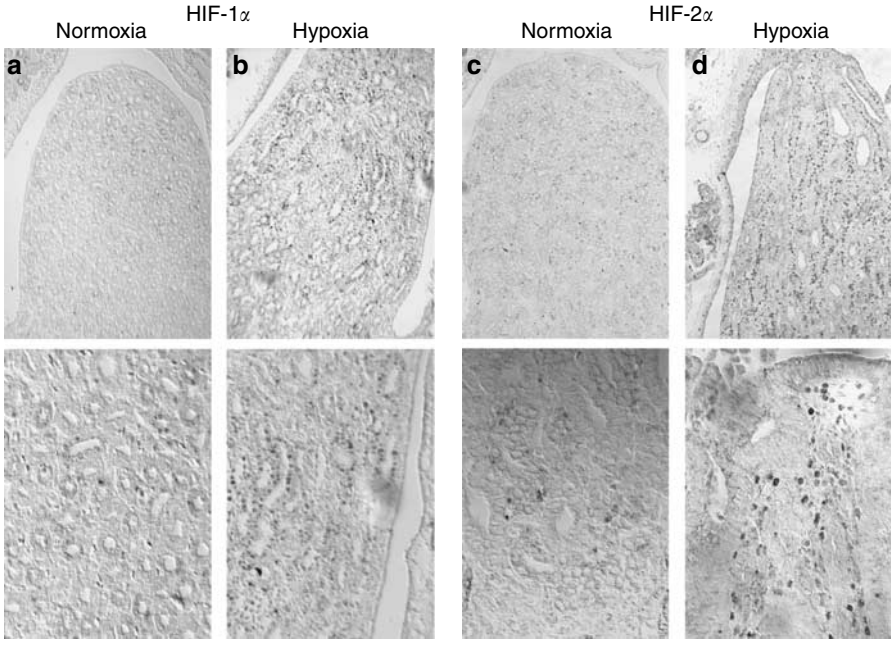

Figure 6 | Induction of HIFα protein by systemic hypoxia in developing rat kidneys. There is a marked increase in nuclear HIF-1α expression of medullary collecting ducts in 3-day-old rats exposed to systemic hypoxia (**b**, 8 vol% O_2 for 4.5 h), as compared to controls (normoxia, **a**). In parallel, compared to normoxia (**c**), HIF-2α accumulation strongly increases in medullary interstitial cells under hypoxic conditions in the developing rat kidney (**d**). Original magnification: upper panels × 200, lower panels × 400.

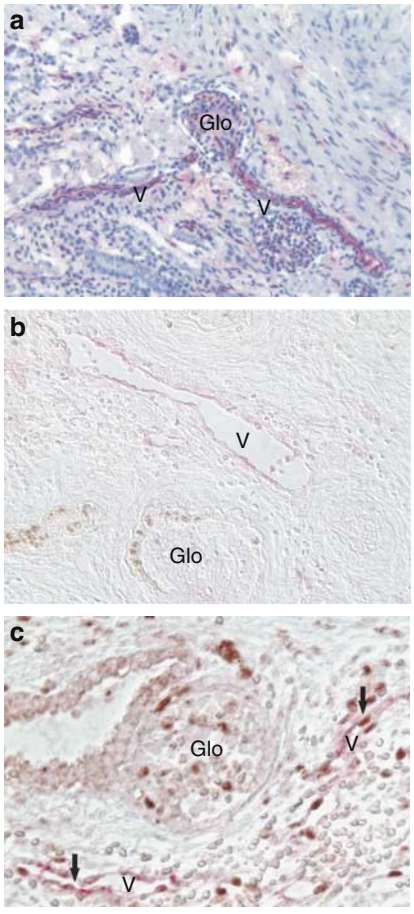

Figure 8 | Localization of the HIF-target gene endoglin in human kidney development, 22nd gestational week. The HIF target gene endoglin (red, fast red staining) is expressed in human kidney development in glomerular and in non-glomerular endothelial (afferent and efferent arteriole) cells (**a**, original magnification × 400). Doublestaining procedures revealed no colocalization of endoglin-positive vessels (red, fast red staining) and HIF-1α-positive glomerular cells (brown, peroxidase staining) (**b**, original magnification × 400). In contrast, HIF-2α (brown, peroxidase staining) colocalized with endoglin (red, fast red staining) in endothelial cells (**c**, × 400, black arrows). (CD – collecting ducts; Glo – glomerulus; V – vessel; magnification × 1000).

temporally controlled pattern. Marked upregulation of HIFα occurred at every examined stage of renal development and decreased at completion of nephrogenesis. In the fetal human kidney, we found clear nuclear expression of HIF-1α in medullary and cortical collecting ducts and in cells of the developing glomerulus. HIF-2α was detectable in glomerular, endothelial and interstitial cells of the cortex and the medulla. In comparison, HIFα expression of the postnatal rat kidney was lower, but cellular specificity was identical in both species. The following hypotheses are proposed:

Hypoxia has a major regulative function for a coordinated kidney development

Hypoxia is regarded as an important physiological factor to control nephrogenesis.[13] Compared to maternal blood oxygen tensions, fetal blood pO_2 is low.[17] In addition, local blood delivery may be inadequate due to the poorly developed vascular tree. Thus, oxygen supply is often insufficient and can result in tissue hypoxia. Freeburg and Abrahamson[12] recently demonstrated occurrence of relevant hypoxia in kidneys of newborn mice by immunostaining with the bioreductive marker pimonidazole, which indicated severe hypoxia. Hypoxia was confirmed in tubular and collecting duct cells of the renal medulla, whereas the renal cortex exihibited only moderate hypoxia. The striking similarities of HIF-1α and -2α expression in developing kidneys compared to that in adult rat kidneys, which have been exposed to systemic hypoxia,[15] suggest a regulatory role of low oxygen tensions in normal kidney development.

Hypoxia is the physiological stimulus which induces HIF in human and rat kidney development

Although there are other known stimuli that might influence HIF expression,[13] low oxygen tension has been characterized as the major physiological condition to stabilize HIF.[5] To further evaluate the impact of tissue oxygenation on HIF expression in the developing kidney, we experimentally induced systemic hypoxia in newborn rats before analyzing their kidneys. Under these conditions, rat kidney HIFα accumulation was further enhanced, but did not change tissue expression patterns; thus, indirectly indicating lack of oxygen supply as the major stimulus for HIFα expression. Nevertheless, tissues from newborn rats do not underlie the restricted oxygen supply from the placenta, thus systemic oxygenation is expected to be higher. It, therefore, remains unclear to what extent oxygen and other factors, such as growth factors and cytokines, are involved in the HIF activation of the newborn but still developing rat kidney. Comparing the human and rat specimens, HIF-1α expression was concordant in both species. On the other hand, HIF-2α was found in postnatal *rat* kidneys only in medullary interstitial cells and in fetal *human* kidneys in podocytes, endothelial and interstitial cells of the cortex and medulla. As the process of nephrogenesis is generally thought to be rather uniform between mammals, these species-specific differences in HIF-2α expression were unexpected. However, differences in tissue oxygen tension before analysis could have an impact on HIF protein detection. Accordingly, after experimental systemic hypoxia, rat kidney HIF expression was more pronounced and strongly resembled the expression pattern and protein levels observed in human fetal kidneys.

HIF-1α has a major role in tubulogenesis – HIF-2α regulates renal vasculogenesis

In the developing kidney, we found that cell populations expressing HIF-1α and HIF-2α were consistently different with an overlap only in the glomerulus. These findings correspond to reports showing cell-specific expression of the two HIF isoforms in the kidney after systemic hypoxia. In hypoxia, HIF-1α was reported to be predominantly detectable in tubular epithelial cells, whereas HIF-2α accumulation was restricted to interstitial, glomerular and endothelial cells.[15] Because HIF-1α was mainly expressed in medullary and

cortical collecting ducts and in the outgrowing uretric buds, HIF-1α is possibly involved in tubulogenesis. In comparison, expression of HIF-2α in endothelial cells suggests a role in modulating the renal vascularization during renal development.

HIF-2α was found in interstitial cells supporting a recent report, which demonstrate predominant transcriptional regulation of erythropoietin (EPO) by HIF-2α.[18,19] EPO is a known target gene of HIF and is most importantly involved in the regulation of red blood cell production and hematocrit. EPO has been shown to be expressed in interstitial fibroblasts in the adult kidney.[20,21] HIF-2α activation in these embryonic renal fibroblasts could contribute to fetal haematopoiesis by enhanced gene expression of EPO, albeit this function is being predominantly fulfilled by the embryonic liver. Hypothetically, an induction of EPO by HIF-2α may also be of relevance for kidney development as EPO has been shown to be involved in recruiting endothelial progenitor cells and stimulating neovascularization *in vivo*.[22] Other targets activated by HIF-2α in these cells could lead to additional effects.

HIF-1α contributes to early and HIF-2α to late glomerulogenesis

Both HIF isoforms were found in the developing glomeruli, suggesting synergistic regulatory roles in glomerulogenesis. Interestingly, in the course of glomerular development we found that HIF-1α preceded HIF-2α expression: in early stages of glomerulogenesis (S- or comma-shaped bodies), only HIF-1α was detectable whereas in the more mature glomeruli HIF-2α was the predominant isoform. To this end, it could be speculated that HIF-1α is involved in the initiation of glomerular development and HIF-2α has further regulatory functions in the process of vessel and podocyte maturation in later stages of glomerulogenesis.

VEGF is potentially regulated by HIF-1α and endoglin by HIF-2α protein

The HIF-target gene VEGF is known to be an important proangiogenic factor. Mice with disruption of the VEGF gene showed severe impairment of vascular development and died at mid-gestation almost before initiation of metanephric development.[23] VEGF has been shown to be regulated by low oxygen tension in kidneys and to be involved in tubulogenesis and rat metanephric development.[24] In colocalization studies, we found that cytoplasmic VEGF expression coincided with nuclear HIF-1α expression in the glomeruli and medullary collecting ducts. Our results in the rat are in concordance with previous reports in the fetal mouse kidney, where VEGF was found to be expressed in glomerular epithelial and collecting duct cells.[25] Coexpression of HIF-1α and VEGF supports the hypothesis of their regulatory role in tubulogenesis. Because VEGF is a secreted protein, which is known to have chemotactic and paracrine effects, its expression by growing tubular epithelia could initiate vasculogenesis in vicinity to the developing tubuli. To this end, VEGF induction by HIF-1α would support the coordinated blood supply for the growing tubuli.

VEGF overexpression is known to initiate vasculogenesis and angiogenesis, but the induced vessels are immature and leaky.[26] Additional angiogenic factors are important in vascular maturation to form competent vessels.[4] Factors contributing to vessel maturation like tie2 have been reported to be regulated by HIF-2α.[27] Therefore, we analyzed further angiogenic factors known to be regulated by HIF. We found that endoglin colocalized with HIF-2α in endothelial cells of developing kidneys. Endoglin binds to several members of the transforming growth factor-β superfamily regulating cellular proliferation and differentiation of endothelial cells (reviewed in Fonsatti *et al.*[28]). Endoglin has been reported to be regulated by hypoxia and was identified as a HIF target gene.[29] To the best of our knowledge, our data represent the first description of endoglin expression in kidney development. HIF-dependent endoglin induction may be important for vessel formation in nephrogenesis.

CONCLUSION

Overall, this study provides further evidence that hypoxia and the HIFs are important factors in the initiation and regulation of nephrogenesis. The distributional pattern and the colocalization studies support the hypothesis of differential regulatory roles of the two HIFα isoforms: HIF-1α appears to be mainly involved in tubulogenesis, whereas HIF-2α is likely to have an important role in the regulation of vasculogenesis. Both HIFα isoforms seem to be involved in the process of glomerulogenesis.

MATERIALS AND METHODS

Human tissue

Fetal human tissue was obtained after induced abortus of fetuses with different developmental abnormalities but no primary hypoxic or kidney diseases ($n = 6$; Table 1). Kidneys of different developmental stages were immersion-fixed in paraformaldehyde (3%) and embedded in paraffin. The collection and use of the paraffin-embedded human tissue was approved by local ethics committees.

Animals

The study was approved by the institutional review board for the care of animal subjects and was performed in accordance with National Institutes of Health guidelines. Newborn male Sprague–Dawley rats (Winkelmann, Borchen, Germany) were killed at the age of 1, 3, 6, 7 and 14 days post partum. Until the time the animals were killed, the neonates stayed with their mother who had free access to food and water.

Tissue preparation of rat kidneys

For immunohistochemistry, the rats were anesthetized with ether. After the onset of anaesthesia, kidneys were generally perfusion-fixed *in situ*, as described previously[30] via cannulation of the left cardiac ventricle. Perfusion was performed with freshly prepared 3% paraformaldehyde in phosphate-buffered saline (pH 7.4) at 240 mmHg for 1.5 min and at 100 mmHg for 3.5 min and then by sucrose/phosphate-buffered saline to stop fixation. Kidneys were

then removed, cut into slices and shock-frozen in liquid nitrogen-cooled isopentane or processed for paraffin embedding.

Induction of hypoxia in newborn rats

To evaluate whether HIF expression in kidneys of newborn rats is modulated by variation of ambient oxygen and thereby variation of tissue oxygenation, four newborn rats were put together with their mother into an air-tight chamber and were exposed to a gas mixture containing 8 vol% O_2 for 4.5 h, which has been shown to induce HIF in the adult kidney.[15] After defined times, rats were killed immediately by cervical dislocation, kidneys were removed and fixed by immersion in freshly prepared paraformaldehyde solution (3%).

Immunohistochemical analysis

Paraffin or cryostat sections (3 μm) were dewaxed in xylene, rehydrated in a series of ethanol washes, and placed in distilled water before staining procedures. Slides were coated with 3-aminopropyl-tri-ethoxysylane. For detection of HIF isoforms, mouse monoclonal anti-human HIF-1α antibody (α67, 1:10 000; Novus Biologicals, Littleton, CO), and polyclonal rabbit anti-mouse HIF-2α antibodies (PM8 (1:10 000) in human tissue and PM9 (1:10 000) in rat tissue, obtained from two different rabbits immunized with a peptide containing amino acids 337–439 of mouse HIF-2α) were used as described previously.[15,31] Additional primary antibodies were monoclonal mouse anti-rat and -human VEGF (1:10 000; Santa Cruz Biotech, Santa Cruz, CA) and monoclonal mouse anti-human endoglin (CD105, 1:5000; DAKO, Hamburg, Germany). Biotinylated secondary anti-mouse or anti-rabbit antibodies were used. For signal amplification and visualization of HIF-1α, HIF-2α, VEGF and endoglin, a tyramine amplification system (CSA kit, Dako, Hamburg, Germany) based on a streptavidin–biotin–peroxidase reaction was used according to the manufacturer's instructions. Antigen retrieval was performed for 6 min for paraffin-embedded tissue and for 3 min for frozen tissue in the preheated Dako target retrieval solution (TRS, Dako, Hamburg, Germany), using a pressure cooker. All incubations were performed in a humidified chamber. As chromogen for the peroxidase –reaction 3,3′-diaminobenzidine tetrahydrochloride (Dako, Hamburg, Germany) and for alkaline phosphatase-linked streptavidin fast red staining solution (Sigma-Aldrich, Munich, Germany) were used. Controls included samples of normoxic adult rat kidneys or preimmune serum of PM9 and PM8, mouse and rabbit immunoglobulins or omission of the primary antibody. Double staining was performed using the CSA-kit two times consecutively with peroxidase-linked streptavidin for HIF-1α or HIF-2α and alkaline phosphatase-linked streptavidin for VEGF or endoglin on one section. For KI67 staining, a mouse anti-human KI67 antibody was used (clone MIB-1, DAKO, Hamburg, Germany). For signal amplification and visualization, DAKO EnVision® Kit (DAKO, Hamburg, Germany) was used according to the instructions of the manufacturer. Between incubations, specimens were washed two to four times in buffer (50 mM Tris-HCl, 300 mM NaCl, 0.1% Tween-20, pH 7.6). For HIFα staining, samples were processed in parallel with an appropriate positive control (hypoxic adult rat kidney).

Morphometric and signal analysis

Signals were analyzed with a Leica DMRB microscope (Leica, Bensheim, Germany) using differential interference contrast. Photographs were digitally recorded by means of a Visitron system (Visitron, Puchheim, Germany).

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