Renal developmental defects resulting from *in utero* hypoxia are associated with suppression of ureteric β-catenin signaling

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Gestational stressors, including glucocorticoids and protein restriction, can affect kidney development and hence final nephron number. Since hypoxia is a common insult during pregnancy, we studied the influence of oxygen tension on kidney development in models designed to represent a pathological hypoxic insult. *In vivo* mouse models of moderate, transient, midgestational (12% O_2, 48 h, 12.5 dpc) or severe, acute, early-gestational (5.5–7.5% O_2, 8 h, 9.5–10.5 dpc) hypoxia were developed. The embryo itself is known to mature under hypoxic conditions with embryonic tissue levels of oxygen estimated to be 5%–8% (physiological hypoxia) when the mother is exposed to ambient normoxia. Both *in vivo* models generated phenotypes seen in patients with congenital anomalies of the kidney and urinary tract (CAKUT). Severe, acute, early hypoxia resulted in duplex kidney, while moderate, transient, midgestational hypoxia permanently reduced ureteric branching and nephron formation. Both models displayed hypoxia-induced reductions in β-catenin signaling within the ureteric tree and suppression of the downstream target gene, *Ccnd*1. Thus, we show a link between gestational hypoxia and CAKUT, the phenotype of which varies with timing, duration, and severity of the hypoxic insult.

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Congenital anomalies of the kidney and urinary tract (CAKUT) present in 3–6 in 1000 humans, representing one of the most prevalent birth defects in man.[1,2] CAKUT encompasses renal agenesis, duplex kidneys, vesicoureteric reflux, ureteropelvic junction obstruction, hypoplasia, and renal dysplasia, occurring alone or as part of multi-organ syndromes. Although the phenotypic spectrum is broad, identifiable mutations are only present in ~10% of CAKUT patients.[3–5] Known causative gene mutations appear to occur in genes responsible for the formation and interaction of the two key progenitor compartments of the developing kidney, the ureteric bud (UB), which forms the collecting duct system and ureter, or the metanephric mesenchyme, which gives rise to the nephrogenic cap mesenchyme and stromal elements. Indeed, mutations in genes such as *RET*, *TCF2/HNF1*β, *PAX2*, *SIX1*, *SALL1*, and *EYA1* have been identified in CAKUT patients.[3–5] In mouse, genetic perturbation of β-catenin signaling has also been associated with CAKUT-like phenotypes.[6,7] In response to Wnt ligand binding, non-phosphorylated/activated β-catenin can translocate into the nucleus and regulate target gene transcription via binding with TCF/LEF transcriptional mediators. This canonical Wnt/β-catenin signaling is critical for a variety of processes during kidney development, including the initial outgrowth of the UB, growth and branching of the ureteric epithelium,[6–8] cap mesenchyme self-renewal, and nephron induction[9–11] and nephron elongation.[12] Mutation of β-catenin within the nephric duct/UB can result in duplex kidney,[6] renal agenesis,[7] and reduced UB branching. Conversely, overexpression of β-catenin disrupts branching[13] and leads to dysplasia,[14,15] suggesting high sensitivity to dose and timing within this pathway.

CAKUT accounts for ~50% of all cases of childhood end-stage renal disease and patients presenting with hypoplasia/dysplasia are likely to have a reduction in nephron number. Reduced nephron number in turn associates with

hypertension, glomerular hyperfiltration, and renal disease.[16] A number of gestational environmental stressors (maternal low protein diet and maternal glucocorticoid exposure) have also been associated with reduced nephron number,[17–20] although the underlying molecular mechanism is not understood. Given the high proportion of CAKUT patients with no discernable genetic mutation, this raises the possibility of environmental contributors/modifiers to CAKUT.

Intrauterine hypoxia, either chronic or acute, is one of the more common insults to the developing fetus, resulting in fetal growth retardation and specific heart and brain defects.[21–27] Although the fetus normally develops under relative hypoxia (physiological hypoxia), estimated at ~5%–8% O_2 at the tissue level,[28] a further reduction in tissue oxygen tension may place the system at greater risk.[29] Gestational hypoxia can occur due to smoking (~25% of all pregnancies in the United States), exposure to environmental pollutants, maternal anemia, placental insufficiency, cord compression, preeclampsia, drug use, heart/lung diseases, and living at high altitude (~140 million people worldwide).[22,29–32] Although the effects of gestational hypoxia on development remain undefined, these are likely to be modified by genetic background. Indeed, while Notch pathway mutations cause scoliosis in humans and mice, similar defects arise in response to severe, acute gestational hypoxia, with the penetrance and severity modified by genetic background.[33]

Although animal studies suggest that fetal hypoxia can reduce renal blood flow,[34] a definitive effect of gestational hypoxia on kidney development had not been demonstrated. Here we show *in vivo* that severe (5.5%–6.5% O_2), acute (8 h), early (9.5–10.5 dpc) gestational hypoxia results in duplex kidney formation, while moderate (12% O_2), transient (48 h), midgestational (12.5–14.5 dpc) (MTM) hypoxia results in renal hypoplasia, both phenotypes within the CAKUT spectrum. In both models, there was a reduced level of β-catenin signaling, providing a mechanistic link between an environmental stressor and kidney development.

RESULTS

To model severe, acute, early gestational hypoxia, such as might occur with severe vasoconstriction (acute blood loss or cocaine use), we placed pregnant dams at either 9.5 or 10.5 dpc within an oxygen-controlled environment at 5.5%, 6.5%, or 7.5% O_2 for 8 h. In our previous studies on the effect of hypoxia on vertebral development, 5.5% was the lowest O_2 concentration tolerated, while 7.5% was the highest O_2 concentration in which vertebral defects were retained.[33] The gestational timing was equivalent to gestational days 22–28 in the human (Carnegie stage 10–12)[35] coinciding with budding of the UB budding from the nephric duct. Pregnancy continued after the hypoxic insult to 17.5 dpc when embryos were collected for magnetic resonance imaging scanning (Figure 1a). A proportion of embryos from each hypoxic litter developed either unilateral or bilateral duplex kidney (Figure 1b), with the penetrance varying with gestational age at exposure and degree of hypoxia. Whole mount immunofluorescence of 10.5 dpc kidneys after severe, acute, early gestational hypoxia confirmed the formation of multiple ureters extending from the nephric duct (Figure 1c).

To investigate the *in vivo* effect of a more MTM hypoxia, we placed pregnant dams at 12.5 dpc into the hypoxia chamber at 12% O_2 for 48 h. This mimics a transient period at high altitude (http://www.altitude.org/high_altitude.php) or a mild placental insufficiency. Embryos collected at 14.5 dpc showed no apparent developmental delay compared with those from normoxic pregnancies (Figure 2a). Placentas were slightly heavier (6%) in the hypoxic group, resulting in a significant increase in placenta to body weight ratio (Figure 2b and Supplementary Figure S1A online); however, both embryo weight and kidney length were lower in the hypoxic group (Figure 2c and d, and Supplementary Figure S1A online). The increased placenta to body weight ratio ratio suggests hypoxia-induced placental adaptations[36] or increased placental vascularity to maintain placental oxygenation, as has been reported at high altitude.[37–40] In comparison with pups from unaffected dams, hypoxic kidneys showed reductions in ureteric branching and glomerular number (Figure 2e–g and Supplementary Figure S1B online). The placenta can compensate by reducing oxygen consumption to maintain oxygen supply to the fetus;[41] hence, we validated a differential hypoxic insult to the embryo using Hypoxyprobe.[42] Hypoxyprobe forms piminadazole adducts with proteins at an oxygen tension below 10 mm Hg (1%–2% O_2). Hypoxyprobe adducts were only detected in kidneys from hypoxic dams (Figure 2h), indicating that an ambient oxygen tension of 12% results in a tissue level hypoxia of $<2\%$ O_2. We term this pathological hypoxia. Transmission electron microscopy revealed cells with wrinkled nuclear membranes characteristic of hypoxic stress[43,44] at a significantly higher rate in kidneys from hypoxic dams (62.2 vs. 12.0%; $P<0.001$; Figure 2i), whereas cell adherens junctions remained intact (Figure 2j, black arrows).

In a proportion of mice subjected to MTM gestational hypoxia, pregnancy was allowed to continue with dams housed at normoxia (Figure 3). Only a transient minor reduction in maternal weight gain was observed in hypoxic dams compared with controls, suggesting no major contribution of nutritional deprivation to fetal weight changes (Figure 3a).[45] Although pups subjected to hypoxia *in utero* remained smaller than controls, kidney weight was slightly higher relative to body weight (Figure 3b and c, and Supplementary Figure S1C online), suggesting organ sparing. To determine whether the reduced renal branching and nephrogenesis persisted after a return to normoxia, final nephron number was assessed at postnatal day 15 (P15). Glomerular number remained only 75% that of controls (Figure 3d and Supplementary Figure S1C online), indicating that MTM hypoxia results in hypodysplasia with a permanent reduction in nephron number. Taken together, these two *in vivo* models indicate that CAKUT can result from an