Von Hippel-Lindau Acts as a Metabolic Switch Controlling Nephron Progenitor Differentiation

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ABSTRACT

Background Nephron progenitors, the cell population that give rise to the functional unit of the kidney, are metabolically active and self-renew under glycolytic conditions. A switch from glycolysis to mitochondrial respiration drives these cells toward differentiation, but the mechanisms that control this switch are poorly defined. Studies have demonstrated that kidney formation is highly dependent on oxygen concentration, which is largely regulated by von Hippel-Lindau (VHL; a protein component of a ubiquitin ligase complex) and hypoxia-inducible factors (a family of transcription factors activated by hypoxia).

Methods To explore VHL as a regulator defining nephron progenitor self-renewal versus differentiation, we bred Six2-TGCtg mice with VHLlox/lox mice to generate mice with a conditional deletion of VHL from Six2+ nephron progenitors. We used histologic, immunofluorescence, RNA sequencing, and metabolic assays to characterize kidneys from these mice and controls during development and up to postnatal day 21.

Results By embryonic day 15.5, kidneys of nephron progenitor cell–specific VHL knockout mice begin to exhibit reduced maturation of nephron progenitors. Compared with controls, VHL knockout kidneys are smaller and developmentally delayed by postnatal day 1, and have about half the number of glomeruli at postnatal day 21. VHL knockout nephron progenitors also exhibit persistent Six2 and Wt1 expression, as well as decreased mitochondrial respiration and prolonged reliance on glycolysis.

Conclusions Our findings identify a novel role for VHL in mediating nephron progenitor differentiation through metabolic regulation, and suggest that VHL is required for normal kidney development.

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both Six2 and the cofactor CITED1 are initially expressed; however, CITED1 expression becomes downregulated at the beginning of differentiation.2,3,6,7 As nephron progenitors mature, Six2 also becomes downregulated and the cells begin to express differentiation markers indicating commitment to a mesenchyme-to-epithelial transition (MET).4,8 This transition, marked by Wnt4 expression, occurs as CM cells make mature cell-cell contacts and ultimately form functional nephrons.9 The maturing nephron progenitors give rise to the nephron: a complex structure comprising a glomerulus and epithelialized tubules that are connected to the collecting duct system.10

Kidney formation is highly dependent on oxygen concentration11 and previous studies show that low levels of oxygen (hypoxia) can result in renal malformations including nephron deficit.12,13 The kidney initially develops in a relatively hypoxic environment and as the vasculature perfuses, oxygen levels increase.14 This tightly regulated change in oxygenation coordinates with the inductive signals from the UE to drive proliferation and subsequent differentiation of the nephron progenitors. Hypoxia-inducible factors (HIFs) play a large role in mediating cellular responses to oxygen fluctuations. Although there are three HIF isoforms (HIF-1α, HIF-2α, and HIF-3α), HIF-1α is considered the master regulator of developmental responses to hypoxia.15 HIF-1α is known to stimulate angiogenesis and maturation of the vasculature, which drives blood flow into hypoxic areas, thereby increasing oxygen concentration.14,16 Increases in oxygen facilitate HIF-1α’s degradation via recruitment of von Hippel-Lindau (VHL).14,16 A protein component of a ubiquitin ligase complex, if prenatal exposure to pathologic hypoxia occurs in the nephron progenitors, VHL is not recruited to degrade HIF-1α, allowing for its sustained expression and transcriptional activation of genes containing hypoxia-response elements (HREs).11,13

Many confirmed HREs are found in genes that encode glycolytic enzymes,1,11,12,14 High rates of glycolysis allow progenitor cells to rapidly undergo expansion through proliferation and nucleotide generation.15–17 However, as differentiating cells mature, they utilize mitochondrial respiration to maintain energy demands of the cell.17–19 HIF-1α is largely responsible for the metabolic profile of progenitor cells and functions in a dual role by promoting glycolysis as well as suppressing mitochondrial respiration.18,20,21 It has also been shown that nephron progenitors exhibit a gradual, decreased reliance on glycolysis for energy generation early in development. As development progresses, nephron progenitors switch from glycolysis to mitochondrial respiration for energy—mediated by an unknown mechanism—and undergo differentiation. To study the potential role in this metabolic shift of von Hippel-Lindau (VHL), a protein component of a ubiquitin ligase complex, the authors generated nephron progenitor cell-specific VHL knockout mice. In addition to identifying VHL as a critical regulator of nephron progenitors’ metabolic switching, the authors’ findings demonstrate that this switch also plays a large role in the differentiation process, and suggest that VHL is required for normal kidney development.

Significance Statement

Nephron progenitors, the self-renewing cells that give rise to nephrons, are particularly metabolically active, relying primarily on glycolysis for energy generation early in development. As development progresses, nephron progenitors switch from glycolysis to mitochondrial respiration for energy—mediated by an unknown mechanism—and undergo differentiation. To study the potential role in this metabolic shift of von Hippel-Lindau (VHL), a protein component of a ubiquitin ligase complex, the authors generated nephron progenitor cell-specific VHL knockout mice. In addition to identifying VHL as a critical regulator of nephron progenitors’ metabolic switching, the authors’ findings demonstrate that this switch also plays a large role in the differentiation process, and suggest that VHL is required for normal kidney development.

METHODS

Mouse Model

We bred transgenic Six2-TGCg mice [JAX009606; Tg(Six2-EGFP/Cre)1Amc/J]26 with VHL-floxed mice [JAX12933; B6.129S4(C)-Vhltm1Jae/J]27 to generate a deletion of VHL in Six2cre+ cells. The University of Pittsburgh Institutional Animal Care and Use Committee approved all experiments (approval no. 16088935). The genotyping primers for the Six2-TGCg and Vhl alleles are listed in Supplemental Table 1.

Western Blotting

Kidneys were lysed in radioimmunoprecipitation assay buffer (ThermoFisher Scientific) and protein was quantified using a Bradford assay (Bio-Rad, Hercules, CA). Samples were electrophoresed on Bolt 4%–12% Bis-Tris Plus Gels and transferred to 0.2 μm nitrocellulose membranes (ThermoFisher Scientific). Antibodies used include anti-VHL (1:250; Santa Cruz Biotechnology), anti–HIF-1α (1:250; Novus), and anti-α/β-tubulin (1:1000; Cell Signaling Technology). Blots were imaged using a ProteinSimple FluoroChem System (ProteinSimple) and quantified using ImageJ (ImageJ/National Institutes of Health Image).

Histology and Immunostaining

Renal histology was assessed at E13.5, E15.5, postnatal day 1 (P1), and P21. Tissue was fixed in 4% paraformaldehyde and processed in paraffin.28 Samples were sectioned at 4 μm and subjected to hematoxylin and eosin or immunostaining. The following primary antibodies or lectins were used at a 1:100 concentration.
Glomerular number was quantified using the Physical Dissector/Fractionator Combination Method, which involves three-dimensional reconstruction of kidneys as previously described. Three-dimensional reconstruction was performed as described, using a Zeiss Model Axio Imager M1 (Zeiss) microscope with Stereo Investigator Stereology image tracing software (MBF Bioscience).

### Three-Dimensional Reconstruction
E13.5 embryos were collected for three-dimensional reconstructions of kidneys as previously described. Three-dimensional reconstruction was performed as described, using a Zeiss Model Axio Imager M1 (Zeiss) microscope with Stereo Investigator Stereology image tracing software (MBF Bioscience).

### Physical Dissector/Fractionator Combination Method for Glomeruli Counting
P21 kidneys were fixed, processed, and serially sectioned at 4 μm. Samples were subjected to hematoxylin and eosin staining. The nth (reference section) and nth+2 (lookup section) sections were counted for glomeruli as described, using a Zeiss Model Axio Imager M1 (Zeiss) microscope with Stereo Investigator Stereology image tracing software (MBF Bioscience).

### Quantification of P1 Nephron Number
P1 kidneys were fixed, processed, and serially sectioned at 4 μm. Samples were immunostained with anti-Wt1 (1:100; Cell Signaling Technologies), and visualized using a Vectastain ABC Kit (Vector Laboratories). Every 20 sections, Wt1+ structures were identified from consecutive sections using a Zeiss Model Axio Imager M1 (Zeiss) microscope with Stereo Investigator Stereology image tracing software (MBF Bioscience). Glomerular number was quantified using the equation by Cullen-McEwen et al.

### Serum Analysis
P21 mice were anesthetized and subject to cardiac puncture for blood collection. Serum was isolated by centrifugation and samples were analyzed for urea nitrogen (Kansas State University).

### RNA-Sequencing
E17.5 kidneys were dissociated and sorted by FACS for GFP+ cells (Flow Cytometry Core). RNA was extracted using a mirNeasy Mini Kit (QiaGen). The Health Sciences Sequencing Core at University of Pittsburgh Medical Center Children's Hospital of Pittsburgh performed library construction and RNA-sequencing (single-end reads, 75 bp). Bioinformatics quality control was performed using FastQC (version 0.11.5), and adapters were trimmed using BBDiamond from the BBMap software package (version 37.41). Reads were aligned to transcripts assembled from the mm10 genome (GRCm38, GENCODE M17) using the Spliced Transcripts Alignments to a Reference software package (version 2.5.3a). Data analysis was performed using R programming (version 3.4.4). Reads aligned to known transcripts were counted using the Bioconductor GenomicAlignments package (version 1.10.1). Differential expression between kidney samples was calculated using the DESeq2 R package (version 1.18.1). Functional annotation analysis was performed using the DAVID database (release 6.8; https://david.ncifcrf.gov/). Real-Time Quantitative PCR
RNA was extracted using a miRNeasy Mini Kit (QiaGen) from E17.5 kidneys. The Superscript First Strand cDNA kit (Invitrogen) was used for cDNA synthesis and samples were analyzed using a C1000 Thermal Cycler (Bio-Rad) to determine mRNA expression levels (normalized to Rn18s). The following genes were analyzed: Pdgf, Gapdh, Tpi1, Bnip3, Ddit4, Pdk1, Eno1, Scl16a3, Citrd1, Six2, Lef1, Salt1, Osr1, Eya1, Jag1, Sox9, Foxd1, and Wnt4 (Supplemental Table 1).

### Magnetic-Activated Cell Sorting Isolation and Seahorse Extracellular Flux
Nephron progenitors were isolated from P1 kidneys using magnetic-activated cell sorting (MACS; Miltenyi Biotec) and cultured as previously described. Nephron progenitors were subjected to a mitochondrial stress test by flux analysis on a XF24 Extracellular Flux Analyzer (Agilent Seahorse Technologies). XF sensor cartridges and assay medium were prepared as previously described. Metabolic profiling was performed by measuring basal respiration for 25 minutes followed by the sequential injection of the substrates oligomycin (1 μg/ml), cyanide p-trifluoromethoxy-phenylhydrazone (0.3 μM), and rotenone (0.1 μM) with antimycin A (1 μM). Data were normalized to cell number.

### Pyruvate Oxidation
P1 kidneys were homogenized in DMEM without supplementation. Homogenates were incubated at 37°C with uniformly labeled 14C-pyruvate in glass tubes sealed with rubber stoppers fitted with hanging baskets. The baskets held filter paper with potassium hydroxide (1M). After 30 minutes, perchloric acid (0.5M) was introduced and the reactions were incubated at 37°C for 1 hour to collect 14C-CO2. 14C-pyruvate incorporation was measured by scintillation counting and normalized to cellular protein content.

### Mitochondrial Density
Kidneys were digested using 0.3% collagenase to create a single-cell suspension. Then, 1×10^6 cells were stained using 200 nM MitoTracker Red FM (ThermoFisher Scientific). Cells were analyzed on a BD LSRFortessa cell analyzer (BD Biosciences).
Mean fluorescence intensity was determined using FlowJo software (version 10.1).

**Kidney Explants**

E13.5 kidneys were cultured on 0.4 μm polyethylene terephthalate membrane inserts as previously described. One kidney from each embryo was cultured in improved minimal essential media (containing 50 μg/ml transferrin) alone and the other was cultured in media containing 10 μM YN1 inhibitor. 

After 72 hours kidneys were fixed in 4% paraformaldehyde for 1 hour. Kidneys were immunostained with antibodies against Six2 and Jag1, following previously described methods.

**Statistical Analyses**

At least three biologic replicates were used for each experiment. Statistical significance was determined by a two-tailed t test (α=0.05), using GraphPad Prism 7. Where appropriate, data are presented as mean±SEM.

**RESULTS**

**VHL Expression Is Required for Differentiation of Nephron Progenitors**

To determine the functional significance of VHL in nephron progenitors, we conditionally deleted VHL from Six2+ nephron progenitors by crossing transgenic Six2-TGC54 mice to VHLlox/lox mice27 (Figure 1A). This produced VHL wild-type [Six2-TGC+/+, VHLlox/lox (VHLNP+/+)], heterozygous knockout [Six2-TGC+/+, VHLlox/lox (VHLNP+/−)], and homozygous knockout [Six2-TGC−/−, VHLlox/lox (VHLNP−/−)] genotypes. Six2-TGC−/− embryos enabled GFP visualization in VHLNP−/+ and VHLNP−/− animals. Because of a slight hypoplastic phenotype associated with the Six2GFPcre transgene, we used VHLNP−/+ littermate controls for all experiments. VHL protein expression (Figure 1B) and HIF-1α protein stabilization (Figure 1C) in VHLNP+/− and VHLNP−/− kidneys confirmed sufficient knockdown and stabilization, respectively. Moreover, subsequent RNA-sequencing analysis (see below) validated VHL knockout specifically in the nephron progenitors.

Histologic examination of VHLNP−/− kidneys did not reveal any significant alterations in development at E13.5 (Figure 1, D and E). However, by E15.5 the VHLNP−/− kidneys begin to exhibit reduced maturation of the nephron progenitors (Figure 1, F and G). By P1 VHLNP−/− kidneys are smaller and remain developmentally delayed (Figure 1, H and I, Supplemental Figure 1) with fewer nephron progenitor-derived structures (see below). To determine the onset of the developmental phenotype, we performed three-dimensional reconstructions of E13.5 kidneys to evaluate kidney size and maturation, which supported the histologic examination. The number of glomerular structures, renal vesicles, and immature glomeruli were not significantly altered between VHLNP+/− and VHLNP−/− kidneys nor were there any differences in kidney volume, UE volume, or glomerular volume (Figure 2, A−H).

Next, we evaluated the expression of nephron progenitor and renal vesicle markers through immunofluorescence staining of E15.5 and P1 VHLNP+/− and VHLNP−/− kidneys as E15.5 is when we first observe a phenotype and P1 is largely an older progenitor population. Staining against the nephron progenitor marker Six2 with Ncam to mark the nascent nephrons at E15.5 (Figure 3, A and B) and P1 (Figure 3, C and D) revealed that VHLNP−/− kidneys have persistent Six2 expression in Ncam+ structures. Further, Ncam expression was also decreased at both E15.5 and P1, suggesting there may be alterations in cell adhesion between nephron progenitors (Figure 3, A−D). For these reasons, we quantified the number of Six2+ cells per cap at P1 and found an increase (P=0.006; Figure 3I).

Next, we performed immunostaining against Jag1 (differentiating structures) and quantified the number of Jag1+ structures per P1 kidney. We saw a reduction in the number of Jag1+ structures (P=0.008; Figure 3, E−H and J), which further suggests that VHLNP−/− kidneys exhibit decreased differentiation. Additionally, we performed immunostaining for Wt1, Sall1, and Lef1, which support these findings (Supplemental Figure 2).

In particular, persistent Wt1 expression in VHLNP−/+ nephron progenitors was consistent with maintenance of a progenitor-like state (Supplemental Figure 2, A and B). To evaluate differentiation capacity, we performed unbiased, stereological counting of Wt1+ glomeruli at P1 and found a 30% decrease (P=0.01; Figure 4A). Although there is a significant decrease in the number of nephrons, the kidney-to-body weight ratio at P1 was unchanged between VHLNP+/− and VHLNP−/− mice (Figure 4B).

Gene expression analysis of whole E17.5 kidneys showed no differences in several nephron progenitor markers, including Cited1, Sall1, Eya1, Six2, and Osr1 (Figure 4C). Gene expression analysis of the differentiation markers Lef1, Wnt4, and Jag1 revealed significant decreases in expression reflecting an inability to properly undergo differentiation (Figure 4C). There were no changes in Foxd1 (stromal progenitors) or Sox9 (ureteric tip identity) gene expression (Figure 4C). Together, this suggests that VHL does not induce profound alterations during the self-renewal phase and likely plays a larger role in the regulation of nephron progenitors that are poised to exit the progenitor niche.

To explore alterations in proliferation and cell death as a possible cause of decreased differentiation, we performed immunofluorescence staining with anti-phospho-histone H3 and terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling, respectively. There were no differences in proliferation or apoptosis (Supplemental Figure 3, A–P). We also performed cell cycle analysis on P1 nephron progenitors using propidium iodide to investigate potential irregularities in the cell cycle or senescence. Cell-cycle analysis showed no differences in G1/G0, S, or G2/M phases (Supplemental Figure 3, Q and R). Collectively, these data indicate that reductions in nephron number do not likely stem from decreased proliferation, increased cell death, cell...
Figure 1. Conditional deletion of VHL from Six2+ nephron progenitors leads to HIF-1α stabilization and reduced nephron structures. (A) Experimental breeding strategy to obtain successful VHL deletion in nephron progenitors using Six2-TGCtg and VHLlox/lox mice. (B) Whole kidney homogenates from P1 VHLNP+/− and VHLNP−/− mice subjected to Western blotting with antibodies against VHL and α/β-tubulin to confirm VHL deletion; ***P<0.001; VHLNP+/− n=3; VHLNP−/− n=5. Error bars indicate SEM. (C) Whole kidney homogenates from P1 VHLNP+/− and VHLNP−/− mice subjected to Western blotting with antibodies against HIF-1α and α/β-tubulin to confirm HIF-1α stabilization.
cycle dysregulation, or cellular senescence before cessation of nephrogenesis in VHLNP+/- kidneys.

**VHLNP+/- Nephron Progenitors Display Dysregulation of Metabolic Gene Expression**

To better understand the mechanisms underlying how VHL contributes to development, we performed RNA-sequencing on E17.5 GFP+ nephron progenitors using FACS. Hierarchical distance clustering and principal component analysis showed clear grouping of VHLNP+/+ and VHLNP+/- samples (n=4; Supplemental Figure 4, A–C). Variance and expression of distribution were estimated using DESeq2 and minus-average plotting (Supplemental Figure 4, D and E). We observed a downregulation of VHL, confirming efficient deletion of this loxP-flanked fragment by Cre recombinase (Supplemental Figure 5A). Controlling the false discovery rate at 5% and focusing on genes with at least two-fold up- or downregulation, we found that 245 known genes were significantly altered: 222 of these genes were upregulated and only 23 were downregulated in VHLNP+/- nephron progenitors (Figure 5A).

![Figure 2](image_url)

**Figure 2.** VHLNP+/- mice exhibit altered kidney development after E13.5. (A) Three-dimensional reconstruction of E13.5 VHLNP+/+ and VHLNP+/- kidneys showing glomerular structure formation. No significant differences in glomerular structure, renal vesicle formation, or immature glomeruli number. (B) Three-dimensional reconstruction of E13.5 VHLNP+/+ and VHLNP+/- kidneys showing tissue volume. No significant differences in kidney volume, UE volume, or glomerular volume. (C–H) Three-dimensional images depicting all glomerular structures (green), ureteric branching patterns (pink), and a reconstruction of the kidneys. n=4. Error bars indicate SEM. UB, ureteric bud.

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stabilization; *P<0.05; n=4. Error bars indicate SEM. (D and E) Hematoxylin and eosin staining of kidneys sectioned at E13.5 appear developmentally normal. (F–I) Hematoxylin and eosin staining of kidneys sectioned at E15.5 and P1 exhibit hypoplasia and reduced developing glomeruli (arrows).
most dysregulated genes included Bnip3l, Bnip3, Pgm2, Fam162a, Pfkp, Slc2a3, Kbtbd11, Stc2, Adams13, and Mgarp (Supplemental Figure 5B). Functional enrichment analysis using DAVID (http://david.ncifcrf.gov) generated the top most affected Gene Ontology term pathways, which included glycolysis (16 genes; 45.7% of KEGG glycolysis/gluconeogenesis genes; \( P=1.13 \times 10^{-22} \)), cellular response to hypoxia (13 genes; 12.6% of KEGG HIF-1 genes; \( P=6.65 \times 10^{-10} \)), and HIF-1α signaling pathway (12 genes; 11.4% of KEGG HIF-1α genes; \( P=3.78 \times 10^{-8} \)), among others (Figure 5B). HIFs, the main targets of VHL, are known to transcriptionally activate genes containing HREs, several of which were found to be altered, including those associated with glycolysis (Pgk1, Gapdh, and Tpi1), hypoxia response (Bnip3 and Ddit4), and HIF-1α signaling (Pdk1 and Eno1) (Figure 5C, Supplemental Figure 5, C and D) in VHL\(^{NP+/-}\) nephron progenitors. A complete list of dysregulated genes in the HIF-1α pathway can be found in Supplemental Table 2. Additionally, we validated several of the RNA-sequencing data results by real-time quantitative PCR using cDNA from VHL\(^{NP+/-}\) and VHL\(^{NP-/-}\) whole kidneys (Figure 5D).

**VHL\(^{NP-/-}\) Nephron Progenitors Exhibit Decreased Mitochondrial Respiration**

RNA-sequencing data analysis implicated metabolic dysregulation as a potential driver of defective nephron progenitor differentiation in VHL\(^{NP-/-}\) kidneys. To compare overall mitochondrial function, P1 VHL\(^{NP+/-}\) and VHL\(^{NP-/-}\) nephron progenitors were MACS-isolated and subjected to a mitochondrial stress test using a Seahorse XF24 extracellular flux analyzer to measure oxygen consumption.\(^3,22\) Oxygen consumption is an indicator of mitochondrial respiration in the presence of a series of metabolic inhibitors and uncoupling agents.\(^42,45\) Overall oxygen consumption, both before and
after substrate addition, resulted in decreased oxygen consumption rate (OCR) (Figure 6A). Basal oxygen consumption was significantly decreased in VHLNP−/− nephron progenitors compared with VHLNP+/− littermate controls (Figure 6B). Moreover, mitochondrial ATP-linked respiration was significantly decreased (Figure 6C). Nonmitochondrial respiration was also calculated by averaging the oxygen consumption after addition of rotenone/antimycin A and was increased in VHLNP−/− nephron progenitors (P=0.02). This indicates decreased reliance on the mitochondria for energy production. P1 VHLNP−/− kidneys also exhibited decreased mitochondrial pyruvate oxidation to CO2 (Figure 6D), further confirming decreased mitochondrial metabolism. To better understand the decrease in mitochondrial respiration, we analyzed mitochondrial density by flow cytometry using MitoTracker Red. There were no significant differences at P1 in mitochondrial density in the GFP+ cell population (Figure 6E). This data indicates that P1 VHLNP−/− nephron progenitors exhibit decreased dependency on mitochondrial ATP for energy production, which is not due to mitochondrial insufficiency.

**VHLNP−/− Nephron Progenitors Exhibit Increased Glycolysis**

In addition to oxygen consumption, the Seahorse XF24 extracellular flux analyzer monitors the extracellular acidification rate, as an indicator of anaerobic glycolysis. VHLNP−/− MACS-isolated nephron progenitors demonstrated increased glycolysis both before and after substrate administration (Figure 7A). Basal extracellular acidification rate was increased (Figure 7B). Moreover, the glycolytic response to oligomycin (mitochondrial respiration inhibitor) was significantly higher in VHLNP−/− nephron progenitors compared with VHLNP+/− nephron progenitors demonstrating increased glycolytic capacity (Figure 7C). We evaluated glycolytic dependence by culturing E13.5 kidney explants in media alone or media containing 10 μM Y1N (glycolysis inhibitor) for 72 hours. Explants were then subjected to immunostaining against Six2 and Jag1 to mark the nephron progenitors and developing structures, respectively. Visual identification of the number of differentiating structures revealed (1) VHLNP−/− kidneys cultured in Y1N were capable of differentiating whereas VHLNP−/− kidneys cultured in media alone underwent minimal differentiation, and (2) VHLNP+/− and VHLNP−/− kidneys cultured in Y1N1 differentiated similarly (Figure 7, D–G). This suggests that the differentiation phenotype arises from an inability of VHLNP−/− nephron progenitors to metabolically switch from glycolysis to mitochondrial respiration during nephrogenesis, further validating the importance of VHL during kidney development.

**VHL Deletion Results in Abnormalities of Juvenile Mouse Kidneys**

VHLNP−/− mice experience premature death around P30; therefore we characterized the renal phenotype at P21.
To assess renal structure and function of the juvenile (P21) mice, we performed gross and histologic examinations, which revealed smaller kidneys with fewer glomerular structures (Figure 8, A–D, Supplemental Figure 6, A and B). The VHLNP<sup>2/2</sup> kidneys appeared to have decreased glomeruli and slight dilation of the proximal tubules (Figure 8, A–D), which is likely owing to a compensatory mechanism to overcome the reduction in nephron number. In fact, we found that the VHLNP<sup>2/2</sup> kidneys have a 50% reduction in nephron number (P<0.001; Figure 8I). Although VHLNP<sup>2/2</sup> kidneys were smaller than VHLNP<sup>+/2</sup> kidneys, the kidney-to-body weight ratio was not different (Figure 6). Additionally, BUN concentration was significantly increased in VHLNP<sup>2/2</sup> mice (Figure 8J), which we hypothesized was due to glomerular insufficiency and not a result of tubular malfunction. To evaluate this hypothesis, we performed immunostaining against Glut1 (glycolysis) and Pck1 (gluconeogenesis), which did not reveal differences in protein expression between the VHLNP<sup>+/2</sup> and VHLNP<sup>2/2</sup> kidneys (Figure 8, E–H), suggesting that the developed tubules are functional.

To determine whether the defect we observed was specific to nephron progenitors or due to a defect in their differentiation into renal vesicles, we generated a mouse model with a conditional deletion of VHL specifically in the renal vesicles utilizing a Wnt4creGFP<sup>47</sup> using the same breeding strategy as described for our VHLNP<sup>+</sup> model above. Interestingly, we did not see significant alterations in body weight, kidney weight, or kidney histology (Supplemental Figure 7 and data not shown) between heterozygous (VHLRV<sup>+</sup>/2) and homozygous (VHLRV<sup>2/2</sup>) mice. This suggests that VHL is dispensable in

Figure 5. RNA-sequencing reveals genetic dysregulation in isolated VHLNP<sup>+/2</sup> and VHLNP<sup>2/2</sup> nephron progenitors. (A) Heat map showing differential gene expression in VHLNP<sup>+/2</sup> and VHLNP<sup>2/2</sup> nephron progenitors. Cutoff for significance was set to LFC >2 and P<0.05. (B) DAVID functional annotation enrichment analysis showing the pathways associated with the gene expression data. (C) Annotation-associated gene expression showing glycolysis gene dysregulation using DAVID analysis (Gene Ontology terms). (D) Real-time quantitative PCR validation of significantly dysregulated genes in the glycolysis, cellular response to hypoxia, and HIF-1α signaling pathways. *P<0.05; RNA-sequencing n=4; real-time quantitative PCR validation n=3. Error bars indicate SEM. FDR, false discovery rate; LFC, log fold change; TPM, transcripts per million.
the differentiated renal vesicle, and thus also dispensable for nephron function. Taken together, we show that VHLNP2/2 mice have malformations resulting in fewer glomeruli and reduced renal function, which is not attributed to a postnatal defect and therefore results from the abnormal development of the nephron progenitors.

DISCUSSION

From our study it is clear that the VHL/HIF pathway plays a major role in differentiation decisions during nephron progenitor maturation. Our data reveals a previously unknown role for VHL in mediating metabolic switching during kidney development that is broadly applicable to other developing systems. Mechanistically, our data suggests that VHL is required for nephron progenitors to adapt to increasing oxygenation and, in response, alter their primary metabolism from glycolysis to oxidative phosphorylation. Moreover, we propose this metabolic switching is an essential process for differentiating nephron progenitors. Specifically, nephron progenitors lacking VHL exhibit upregulated glycolysis and downregulated mitochondrial respiration. Although mitochondrial respiration was suppressed, mitochondrial density did not differ between VHLNP+/− and VHLNP−/− nephron progenitors. We conclude that the mitochondria are healthy and retain the ability to function normally; however, they lack substrate (i.e., pyruvate) because of the HIF-mediated upregulation of PDK, which effectively blocks pyruvate utilization by the mitochondria. Instead of pyruvate shuttling into the mitochondria, it is preferentially converted into lactate and recycled back into the glycolytic pathway. The profound metabolic alteration leads to decreased differentiation indicated by reduced nephron numbers at both P1 and P21. This model simulates pathologic hypoxia, which has previously shown to cause a reduction in nephrons13; however, the underlying mechanisms have not been previously identified.

It is well known that kidney formation is highly dependent on oxygen concentration, which is largely regulated by VHL and HIFs. Of particular interest is the fact that VHLNP−/− nephron progenitors have access to oxygen, but remain unable to respond to the oxygen. We hypothesize that the VHLNP−/− differentiation phenotype is due to prolonged maintenance of a progenitor niche, as demonstrated by persistent Six2 and Wt1 expression and decreased nephron number. Six2-expressing cells represent a multipotent nephron progenitor population expressed throughout kidney development,48,49 and Six2 must be downregulated to initiate MET.49,50 Furthermore,
another study has shown that HIF-1α blocks MET, which serves as another potential mechanism leading to decreased nephron number. Although our model has a significant reduction in nephron number, our data suggests that either a subset of nephron progenitors remain capable of maturing into nephrons or that the differentiation process is not as efficient in VHLNP²/² mice. Similarly, when VHL is deleted from Foxd1-expressing stromal progenitors, the resulting offspring exhibit nephron deficit and postnatal death by 14 days of age, which was attributed to increased HIF expression. Our data shows that the nephrons that do form are either derived from nephron progenitors that underwent differentiation early (before E15.5) or due to inefficient nephron progenitor differentiation. The VHLNP²/² mice do not exhibit a profound phenotype until E15.5, which we hypothesize is because of the physiologically hypoxic environment the kidneys are subjected to in early development, before VHL is critical. Therefore in the absence of VHL before E15.5, the nephron progenitors are capable of normal differentiation; however, after this time they fail to undergo sufficient differentiation. A previous study by Wang et al. also investigated renal structure and function in Six2-creEGFP-VHL null mice for the purpose of studying renal cell carcinoma. Although our model exhibits profound abnormalities, their mice are histologically normal and do not exhibit any malformations or dysfunction until approximately 4 months of age. We see an accelerated phenotype, which we attribute to differences in mouse background strains.

Another significant function of VHL-mediated HIF degradation is the regulation of metabolism. Previous interrogations of HIF implicate it in the regulation of glycolysis and oxidative phosphorylation. Although VHL has not been interrogated as a regulator of metabolism in nephron progenitors, HIF-1α is a known mediator in other cell types, suggesting that VHL must also play an important role in regulating the metabolic profile of these progenitors. Moreover, VHL-related cancers exhibit similarly decreased mitochondrial respiration in favor of glycolic metabolism even in the presence
of oxygen, a phenomenon known as the Warburg effect.\(^{57-59}\) Although our \(VHL^{NP-/-}\) model is not a cancer model, these mice do exhibit severe alterations in the nephron progenitor metabolic profile consistent with the Warburg effect. In addition to enhanced glycolysis, a previous study investigated the metabolic profiles of E13.5 and P0 nephron progenitors and showed that younger progenitors demonstrate a higher dependence on glycolysis whereas older progenitors are more dependent on oxidative phosphorylation.\(^{22}\) Our study suggests that the phenotype associated with the \(VHL^{NP-/-}\) mice can be attributed to metabolic alterations leading to an inability of the cells to transition from glycolysis to oxidative phosphorylation.

In summary, our study suggests that VHL is crucial for the metabolic switching required during progenitor cell differentiation, and validates that strict regulation of oxygenation is vital for cellular differentiation. Thus, VHL is also an essential mediator dictating proliferative, poised, and differentiating nephron progenitors and likely plays a critical role in cell fate decisions. Additional mechanistic interrogation to describe the roles of VHL and HIFs in anaerobic glycolysis, mitochondrial respiration, and cell fate are ongoing.

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Cargill designed and performed experiments, provided intellectual input, performed data analysis, and wrote the entirety of the manuscript. Dr. Hemker, Dr. Murali, Dr. Mukherjee, Dr. Bushnell, Dr. Bodnar, and Dr. Liu conducted experiments and provided intellectual input. Dr. Clugston and Dr. Kostka performed RNA-sequencing data analysis and wrote RNA-sequencing methods. Dr. Hemker, Dr. Bates, Dr. Ho, Dr. Saifudeen, Dr. Goetzman, and Dr. Sims-Lucas edited the manuscript and provided intellectual input to the project. All authors approved the final version of this manuscript.

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DISCLOSURES

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SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2018111170/-/DCSupplemental.

Supplemental Figure 1. P1 renal histology reveals smaller kidneys with fewer glomeruli.

Supplemental Figure 2. VHL<sup>NP</sup>−/− kidneys remain in an early progenitor state with decreased differentiation.

Supplemental Figure 3. VHL<sup>NP</sup>−/− kidneys do not exhibit alterations in proliferation or apoptosis across development.

Supplemental Figure 4. RNA-sequencing gene expression clustering.

Supplemental Figure 5. RNA-sequencing gene expression.

Supplemental Figure 6. VHL<sup>NP</sup>−/− kidneys are significantly smaller than VHL<sup>Np</sup>+/− kidneys.

Supplemental Figure 7. Wnt4VHL kidneys do not exhibit any histological differences by P21.

Supplemental Table 1. List of primers for real-time quantitative PCR and genotyping.

Supplemental Table 2. List of genes dysregulated in the HIF-1α pathway.

REFERENCES


