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# Hepatic Vascular Tumors, Angiectasis in Multiple Organs, and Impaired Spermatogenesis in Mice with Conditional Inactivation of the *VHL* Gene[1]

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## ABSTRACT

**von Hippel-Lindau (VHL) disease is a multisystem inherited cancer syndrome characterized by the development of highly vascular tumors including hemangioblastomas of the retina and central nervous system, pheochromocytomas, and clear cell renal carcinoma, which result from somatic inactivation of the wild-type *VHL* allele in cells harboring a germ-line *VHL* mutation. Homozygous inactivation of the *VHL* gene in mice resulted in embryonic lethality. To produce a mouse model that closely mimics human VHL disease and avoids embryonic lethality, we used Cre/lox site-specific recombination technology. We generated mice carrying conditional *VHL* alleles and a *cre* transgene under the control of the human β-actin promoter, which directs *cre* expression in a mosaic pattern in multiple organs. *VHL*$^{f/d}$*/Cre* mice developed multiple, hepatic hemangiomas that led to premature death, as well as angiectasis and angiogenesis in multiple organs. Interestingly, testes of male *VHL*$^{f/d}$*/Cre* mice were unusually small with severely reduced sperm count resulting in infertility. Loss of pVHL function in this *VHL* conditional knockout mouse model results in an extensive abnormal vascular phenotype in multiple mouse organs, which will provide a useful animal model for testing potential antiangiogenic therapies for VHL disease treatment. Importantly, the phenotypic defects in sperm development observed in these mice support a novel role for *VHL* in spermatogenesis. This *VHL* conditional knockout mouse model will provide an *in vivo* system for studying the functional requirement of the *VHL* gene in reproductive biology.**

## INTRODUCTION

VHL[3] disease is an inherited, autosomal dominant disorder characterized by a predisposition to develop tumors in multiple organs, including hemangioblastomas of the CNS and retina, clear cell renal carcinomas, pheochromocytomas, cysts and neuroendocrine tumors in the pancreas, inner ear endolymphatic sac tumors, cystadenomas of the epididymis, and tumors of the broad ligament (1–3). The *VHL* tumor suppressor gene, located at chromosome 3p25, was discovered by positional cloning (4), and mutations in the *VHL* gene were identified in the germ line of VHL patients with loss of the wild-type allele in the majority of VHL-associated tumors, supporting the tumor suppressor gene "two hit hypothesis." (5–7). Additionally, *VHL* was mutated in 57% of sporadic clear cell renal carcinoma, with concomitant loss of the wild-type allele (8), in 25–50% of sporadic hemangioblastomas of the CNS (9, 10), and in several cystadenomas of the epididymis (11). The spectrum of tumors in VHL families is variable (12), and phenotype-genotype correlations have led to additional subclassification of the VHL disease (7, 13, 14).

Extensive experimental evidence supports the role of pVHL as the substrate receptor of a ubiquitin ligase (E3) multiprotein complex (15–19). Under normoxic conditions, the E3 ligase complex targets HIF-α subunits for ubiquitin-mediated degradation (20–23). In the absence of functional pVHL, HIF1-α and HIF2-α accumulate, even in the presence of oxygen, resulting in elevated transcription of a wide variety of HIF-controlled genes (24–27). Overproduction of these angiogenic factors is thought to contribute to the highly vascular tumors that develop in VHL patients. However, a novel *VHL* mutation that dysregulates HIF1-α and its downstream target genes without causing tumors to develop in patients (28) suggests the possibility that another target gene(s), alone or in combination with HIFα, may participate in tumorigenesis.

Most germ-line *VHL* mutations compromise the binding of the elongins through the α-domain of pVHL or the binding of HIFα through the β-domain (15, 19, 21). In the kidney parenchyma from VHL patients, *HIF* activation was shown to be an early event occurring in morphologically normal single cells within the renal tubules after *VHL* inactivation (29), leading to progressive up-regulation of the HIF pathway and tumorigenesis. The VHL protein has been shown recently to bind to and stabilize microtubules, protecting them from depolymerization *in vivo* (30). Additionally, pVHL may play a role in the formation of a mature extracellular fibronectin matrix (31, 32).

Several groups have attempted to develop a mouse model that mimics the phenotypic features of VHL disease. Mice homozygous for the *VHL* null allele died *in utero* because of vascular abnormalities of the placenta (33), but heterozygous *VHL* null mice on a C57BL/6 background were phenotypically normal. Haase *et al.* (34) used Cre/lox site-specific recombination to avoid embryonic lethality, using an albumin promoter-driven Cre recombinase to conditionally inactivate the *VHL* gene in the livers of BALB/c mice. Homozygous deletion of *VHL* in the liver resulted in enlarged blood-filled vascular cavities, severe steatosis, and foci of increased vascularization in the liver parenchyma causing death at 6–12 weeks. Hepatic hemangiomas developed in the livers of heterozygous *VHL* null mice on a BALB/c background; however, no phenotype was observed in other organs.

The hallmark of human VHL disease is the development of multifocal, highly vascular tumors in VHL target organs. These tumors are thought to arise in cells carrying a germ-line *VHL* mutation as a consequence of independent somatic mutations or deletions that inactivate wild-type *VHL*. Indeed, loss of heterozygosity studies of the kidney and CNS of VHL patients have revealed numerous microscopic foci of dysplastic cells, which exhibit inactivation of the wild-type *VHL* allele (35). In an effort to produce a *VHL* conditional knockout mouse model that more closely mimics human VHL dis-

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[3] The abbreviations used are: VHL, von Hippel-Lindau; f, *VHL* floxed allele; d, *VHL* deleted allele; +, *VHL* wild-type allele; *Cre,* human β actin Cre recombinase transgene; *CreER*$^{TM}$, tamoxifen-inducible Cre recombinase-mutant estrogen receptor fusion protein transgene; CNS, central nervous system; p, protein; HIF, hypoxia-inducible factor; ES, embryonic stem; floxed, flanked by *lox*P sites; VBP1, von Hippel-Lindau binding protein-1.

ease, we have used a human β-actin promoter-driven *cre* transgenic mouse that expresses *cre* in a mosaic pattern in multiple organs. Homozygous inactivation of *VHL* in numerous cells of multiple target organs in the mouse mimics the numerous, independent somatic mutations, which occur in human VHL disease. We present the phenotype of the *VHL* conditional knockout mouse model generated by this approach, including hepatic hemangiomas, angiectasis, and angiogenesis in multiple organs, and defective spermatogenesis, which represents a novel role for the *VHL* gene in reproductive biology.

## MATERIALS AND METHODS

**Generation of *VHL*-floxed Mice.** A murine *VHL* genomic clone containing intron 1, exons 2 and 3, and the 3′ untranslated region of the *VHL* gene was isolated from a 129/SVJ mouse λ phage library (Stratagene, La Jolla, CA) and subcloned into pBluescript vector. A loxP-*neo* -loxP cassette was inserted in an *Eco*RI site upstream of exon 2 for G418 selection, and a second loxP site was inserted in a unique *Hind*III site, 5.8 kb downstream, destroying the *Hind*III site. A thymidine kinase gene was inserted into the multiple cloning site of the *VHL* target vector for negative selection against random insertion. The vector was linearized with *Not*I and electroporated into strain129-derived mouse ES cells. ES cell clones, which had undergone homologous recombination with the *VHL* target vector, were selected on G418 (250 μg/ml) and gancyclovir (2 μM) -containing medium, using standard procedures (36). Subsequently, the *neo* cassette was deleted from the correctly targeted ES cells by transient transfection with a cytomegalovirus promoter-driven Cre recombinase expression vector. *Hind*III-digested DNA from ES cell clones was analyzed by Southern blot analysis using a PCR-generated 250-bp external probe, located upstream of the 5′ loxP site, which detected a 7.7 kb *Hind*III fragment for the *VHL* wild-type allele, an 18.2 kb *Hind*III fragment for the *VHL* floxed allele (without *neo* gene), and a 12.4 kb *Hind*III fragment for the *VHL* deleted allele (Fig. 1, *A* and *B*).

The targeted 129/SVJ ES cells, in which exons 2 and 3 of *VHL* were floxed, were injected into C57BL/6 blastocysts, and chimeras were selected that showed germ-line transmission of the *VHL* floxed allele when backcrossed to C57BL/6 mice (37). The mice were genotyped by Southern blot analysis of *Hind*III-digested tail DNA using the external probe and strategy described above.

**Evaluation of Cre Expression in β-Actin *cre*-transgenic Mice.** To generate the *VHL* conditional knockout mice, we selected a transgenic mouse line on a C3H background, in which Cre recombinase expression was under the control of a human β-actin promoter (38).[4] Animal care was provided in accordance with the procedures outlined in the "Guide for the Care and Use of Laboratory Animals" (NIH Publication No. 86-23, 1985). Tissue expression of β-actin promoter-driven Cre recombinase was evaluated in offspring from crosses of the β-actin *cre* transgenic mice with ROSA26 reporter mice (39). Cre recombinase expression in tissues of ROSA 26 reporter mice will delete the floxed *neo* expression cassette upstream of the *LacZ* gene, permitting *LacZ* gene expression and detection by standard β-galactosidase staining techniques.

**Delivery of Cre Recombinase by Tamoxifen Induction of *CreER*$^{TM}$ Mice.** *Cre ER*$^{TM}$ mice carry a transgene encoding a fusion protein between Cre recombinase and the ligand-binding domain of a mutant mouse estrogen receptor, which binds the synthetic ligand tamoxifen, but not endogenous17β-estradiol (40). The CreER$^{TM}$ fusion protein is sequestered in the cytoplasm until administration of tamoxifen, which allows nuclear localization of Cre-ER$^{TM}$ and deletion of the floxed *VHL* allele. Offspring produced from a cross between female *VHL* $^{f/f}$ and male *VHL* $^{d/+}$*/CreER*$^{TM}$ mice were injected i.p. with tamoxifen in corn oil (0.36 mg/g body weight) or corn oil alone at 10 weeks of age. Animals were euthanized if found moribund. Autopsies were performed to evaluate the organs.

**Delivery of Cre Recombinase by Adv/cre Injection.** A recombinant adenoviral vector that contained the *cre* gene under control of the herpes simplex virus thymidine kinase promoter (Adv/cre) was a gift from Dr. Leslie Krushel (Scripps Research Institute, La Jolla, CA; 41). Virus stocks were prepared as described (41) and titered on 293 cells. Nine *VHL* $^{f/d}$ and 6 *VHL* $^{f/+}$ mice were injected i.v. in the tail vein with $8 \times 10^8$ plaque-forming units of Adv/cre. Animals were euthanized at 3-month time points and autopsied for phenotype evaluation.

**Production of *VHL*-conditional Knockout Mice.** Heterozygous *VHL* floxed mice (*VHL*$^{f/+}$) were intercrossed to generate homozygous *VHL* floxed mice (*VHL*$^{f/f}$) and crossed with β-actin *cre* transgenic mice (*Cre/Cre*) to produce mice with one *VHL* deleted allele and a human β-actin *cre* transgene (*VHL*$^{d/+}$*/Cre*). To generate *VHL* conditional knockout mice, we crossed *VHL*$^{f/f}$ mice with *VHL*$^{d/+}$*/Cre* mice and selected for *VHL* $^{f/d}$*/Cre* offspring by Southern blot analysis of *Hind*III-digested tail DNA.

**Survival Rate Analysis.** We recorded survival data for all four of the genotypes and introduced the data into the SAS ver.8.0 statistical program for analysis.

**Phenotype Evaluation and Histopathology.** Phenotype evaluation was performed on animals from 4–12 months of age. Mice were euthanized when moribund, and age-matched controls were sacrificed at the same time. Body and organ weights were recorded during a complete necropsy. Tissues were fixed in 10% formalin, embedded in paraffin, and 5-μm sections were prepared. The sections were deparaffinized and rehydrated through an ethanol dilution series into water. Sections were stained with H&E.

**Immunohistochemistry.** Selected tissues were frozen in OCT with liquid nitrogen and sectioned at a thickness of 10 μm. The sections were fixed in methanol:acetone (1:1) for 10 min at −20°C. Monoclonal anti-HIF-1α (residues 432–528; 1:200; NeoMarkers) was used as the primary antibody for immunohistochemistry. Detection was performed with the UltraVision Mouse Tissue Detection System (NeoMarkers).

**Western Analysis.** Mice were killed by $CO_2$ asphyxiation. Liver and testes were collected, immediately snap frozen in liquid nitrogen, and stored at −80°C. Whole-cell extracts were prepared by homogenization and lysis in 20 mM HEPES, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 1 mM $Na_3VO_4$, leupeptin 10 μg/ml, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS. The homogenate was centrifuged at 2000 rpm for 2 min at 4°C, and the supernatant was stored at −80°C. Total protein was determined with BCA protein assay reagent (Pierce). VHL protein (250 μg/sample per reaction) was immunoprecipitated with monoclonal antihuman VHL Ig32 (1 μg antibody per reaction; PharMingen), separated by 14% SDS-PAGE, and transferred to a nitrocellulose membrane (Millipore). The membranes were blocked with 5% skim milk in 0.1 M PBS buffer (pH 7.4). After incubation with antimouse VHL M-20 antibody (1:400; Santa Cruz Biotechnology), the membrane was washed with PBS for $3 \times 10$ min, and then incubated with horseradish peroxidase-conjugated secondary antibody (Amersham Pharmacia Biotech). Signals were detected using the enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech). To monitor for equal protein loading, β-actin levels in cell lysates were evaluated by Western blot analysis.

**Infertility Testing.** Eight 10–15-week-old *VHL*$^{f/d}$*/Cre* males were bred with 16 age-matched C57BL/6 females over a 4-month time period to evaluate infertility.

**Sperm Counts.** The right epididymis was placed into PBS (0.5 ml), the sperm was released with a 27 1/2-gauge needle, and incubated at 37°C in a 5% $CO_2$ atmosphere for 10 min. The sperm were loaded into chamber slides and counted under a dark field microscope with a computer-assisted sperm analysis program (Hamilton-Thorne Research)

## RESULTS

**Generation of Mice with a *VHL*-targeted Allele by Cre/lox Site-specific Recombination.** To generate a conditional knockout mouse model for VHL disease, we constructed a *VHL* targeting vector, in which exons 2 and 3 of the mouse *VHL* gene were floxed (Fig. 1*A*). The *VHL* targeting vector was then introduced into mouse ES cells by electroporation, and homologous recombination was achieved at a frequency of 5%. The *neo* gene used for positive selection of correctly targeted ES cells was removed by Cre-mediated recombination, and then mice were generated from these targeted ES cells with germ-line transmission of the *VHL* conditional allele.

[4] M. E. Palko and L. Tessarollo, unpublished observations.

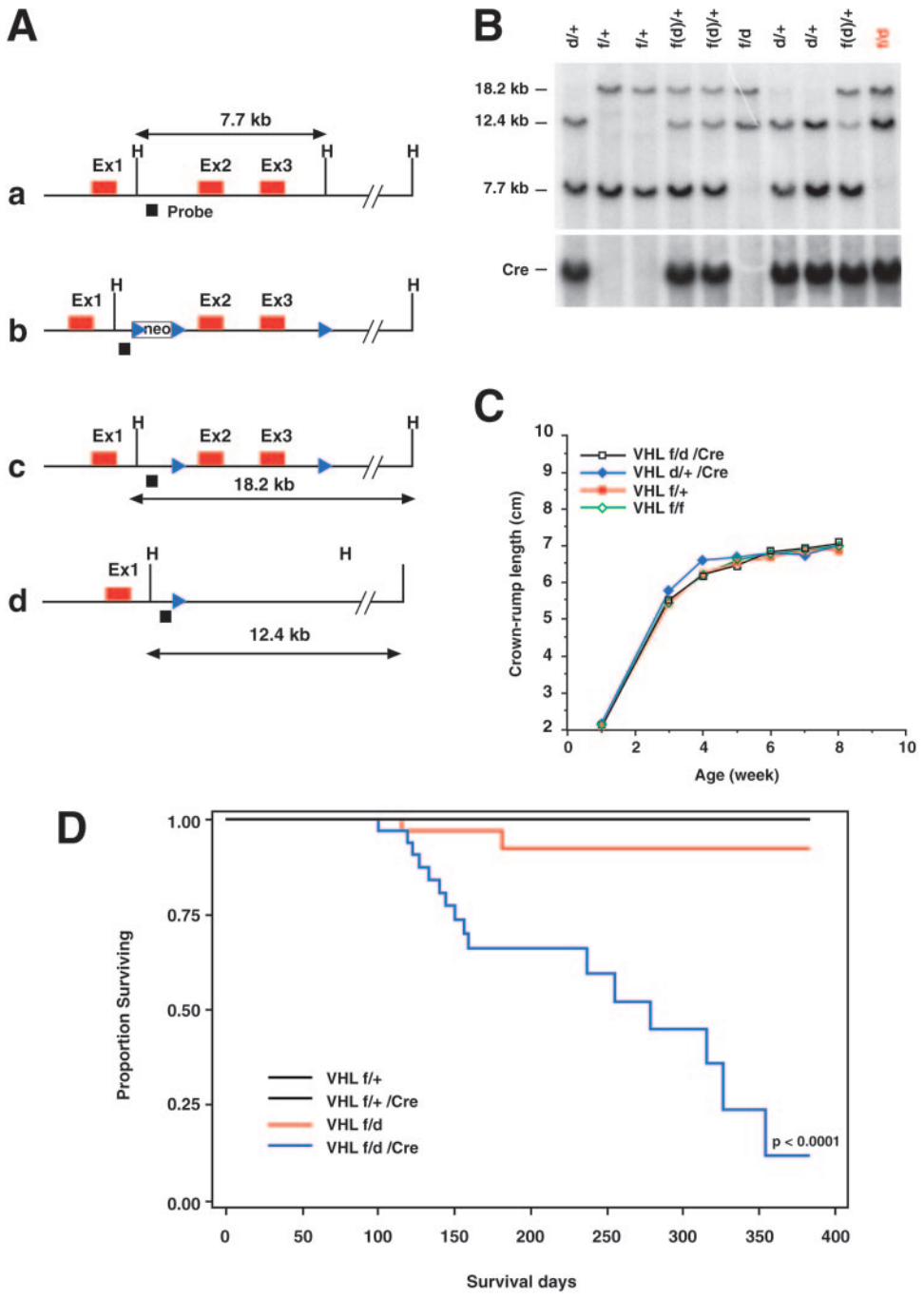


Fig. 1. Generation of a mutant mouse line with conditional inactivation of the *VHL* gene. *A,* targeting of the murine *VHL* locus. *a,* wild-type *VHL* locus. *b, VHL* targeting vector, in which loxP-*neo*-loxP cassette was inserted into intron 1 and a single loxP site replaced a *Hind*III site in the 3′ untranslated region, was introduced into murine ES cells by homologous recombination. After positive ES selection on G418, the *neo* gene was deleted by transient Cre expression *in vitro*. *c, VHL* floxed (*f*) allele in the mutant mice. *d, VHL* deleted (*d*) allele after *in vivo* Cre expression in mutant mice. The 5′ external probe was used to detect *Hind*III fragments of 7.7kb (*wt*), 18.2kb (*floxed allele*) and 12.4kb (*deleted allele*) by Southern blot analysis. Abbreviations: *H, Hind*III. Symbols: loxP, *blue triangle;* 5′ external probe, *black box; VHL* exons, *red boxes*. *B,* Southern blot analysis of *Hind*III-digested mouse tail DNA from mutant mice with different genotypes. The 5′external probe detected the wild-type *VHL* (+) allele (7.7 kb), the *VHL* floxed (*f*) allele (18.2 kb), and the *VHL* deleted (*d*) allele (12.4 kb) after expression of the β-actin promoter-driven *Cre* transgene (top *panel*). Blots were stripped and reprobed with a PCR-generated *Cre* probe derived from the *Cre* coding sequence (*bottom panel*). Mosaic β-actin promoter-driven Cre recombinase expression can produce incomplete deletion of the floxed allele as seen in *Lanes 4, 5* and *9*. *Lane 10* represents the genotype from a conditional *VHL* knockout mouse, which carries floxed and deleted *VHL* alleles and the *Cre* transgene, and developed a vascular phenotype. *C,* growth curves for mutant mice with different *VHL* genotypes. Crown-rump length was measured over an 8-week period for mouse littermates with different *VHL* genotypes (18–20 mice/group). No significant differences were observed during this rapid growth period. *D,* survival curves of mutant mice with different *VHL* genotypes. Twenty-two mice of each *VHL* genotype were evaluated for survival over 1 year. Data were analyzed using the SAS program ver. 8.0. Over 90% of $VHL^{f/d}$/*Cre* mice died by 1 year; mice with other *VHL* genotypes maintained >90% survival rate.

**Evaluation of β-Actin Promoter-driven Cre Recombinase Activity Demonstrates Mosaic Expression in ROSA26 Mice.** Cre/lox site-specific recombination mediates deletion of the floxed allele of a target gene in a tissue- or time-dependent manner, thus circumventing embryonic lethality, when homozygous deletion of the gene is lethal (42). To produce a mouse model that would closely mimic human VHL disease, we wanted to generate mice in which random *VHL* deletion events occurred in cells carrying a germ-line *VHL* deletion. We obtained a transgenic mouse line with Cre recombinase under the control of the human β actin promoter.[4] In general, the human β-actin promoter is expressed ubiquitously, and it is used to generate transgenic mice expressing high levels of a particular transcript (43). However, for this study we chose a line, which expressed *cre* during early stages of embryogenesis in a stochastic manner, generating an incomplete pattern of Cre expression. When the human β-actin promoter-driven *cre* transgenic line was crossed with the ROSA26 reporter mouse (39), we found that Cre was expressed and mediated recombination with a mosaic pattern in a variety of organs including liver, heart, kidney, lung, pancreas, brain, retina, spleen, and adrenal gland (Fig. 2). Strong expression of human β-actin promoter-driven Cre recombinase was observed only in the testis and epididymis. The mosaic tissue expression pattern of β-actin promoter-driven Cre recombinase provided a system for Cre delivery, which was robust in many of the VHL target tissues, but not ubiquitous, thereby circumventing embryonic lethality.

**Growth Rates of *VHL*-conditional Knockout Mice Were Comparable with Littermates during First 8 Weeks of Life.** Crossing heterozygous *VHL* floxed ($VHL^{f/+}$) mice with β-actin *cre* transgenic

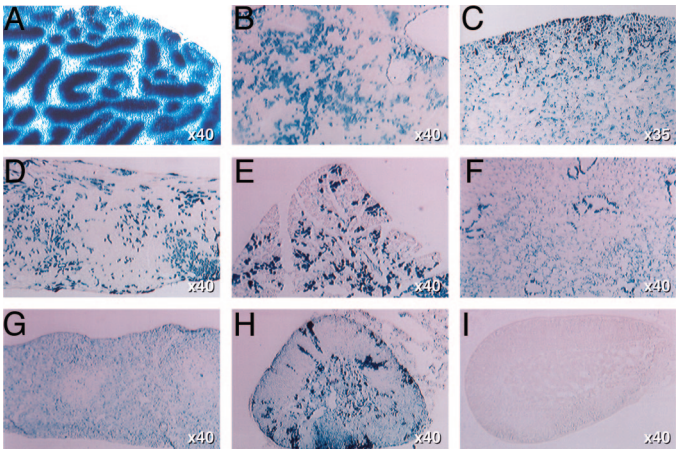


Fig. 2. Expression of the β-actin promoter-driven *Cre* transgene in ROSA26 reporter mouse tissues. Male β-actin *Cre* transgenic mice were bred to female ROSA26 reporter mice, offspring were sacrificed at 3 months, and selected organs were evaluated for Cre-mediated *LacZ* expression by staining for β-galactosidase activity. Positive X-gal staining occurred uniformly in testis (*A*), and mosaically in liver (*B*), kidney (*C*), heart (*D*), pancreas (*E*), lung (*F*), spleen (*G*), and adrenal gland (*H*). Negative X-gal staining is shown for a control tissue (adrenal gland, *I*). Magnification power is given.

mice produced mice with a heterozygous *VHL* deleted allele ($VHL^{d/+}$/*Cre*) when Cre expression occurred in the germ cells and $VHL^{f/+}$/*Cre* mice, when Cre expression did *not* occur in the germ cells, but instead mosaically in developing tissues. Crosses between *VHL* homozygous floxed ($VHL^{f/f}$) mice and $VHL^{d/+}$/*Cre* mice produced $VHL^{f/+}$, $VHL^{f/+}$/*Cre*, $VHL^{f/d}$, and $VHL^{f/d}$/*Cre* mice (*VHL* conditional knockout). Some $VHL^{f/d}$/*Cre* embryos escaped embryonic lethality because of mosaic homozygous inactivation of *VHL* alleles in developing tissues (data not shown). *VHL* alleles and the *cre* transgene were identified by Southern blot analysis of *Hind*III-digested tail DNA (Fig. 1, *A* and *B*).

Comparison of growth curves for $VHL^{f/+}$ mice, $VHL^{f/f}$ mice, $VHL^{d/+}$/*Cre* mice, and $VHL^{f/d}$/*Cre* mice indicated that the presence of a *VHL* floxed allele or deleted allele did not affect growth; these mice grew and developed normally during the rapid growth phase from birth to 8 weeks (Fig. 1*C*).

**VHL Protein Levels Were Reduced in Tissues of Mice with *VHL* Inactivation.** To compare pVHL expression in *VHL* heterozygous ($VHL^{f/d}$) and homozygous deleted mice ($VHL^{f/d}$/*Cre*) with expression in *VHL* heterozygous floxed mice ($VHL^{f/+}$), we evaluated the levels of VHL protein in liver and testis tissues of littermates with different *VHL* genotypes by Western analysis. Two VHL protein products (long and short forms, $M_r$ 21,000 and $M_r$ 19,000, respectively) were identified in liver and testis tissues. Reduced expression levels of VHL protein were detected in $VHL^{f/d}$ mice compared with $VHL^{f/+}$ mice (Fig. 3*A*). As a result of mosaic tissue expression of Cre recombinase, we detected an additional reduction in pVHL expression in $VHL^{f/d}$ mice carrying the *cre* transgene. No differences in pVHL expression between *VHL* wild-type and floxed mice were observed (data not shown).

***VHL*-conditional Knockout Mice Exhibited 50% Mortality at 7 Months.** The $VHL^{f/d}$, $VHL^{f/+}$, and $VHL^{f/+}$/*Cre* control littermates grew and developed normally with survival rates of 100%, 100%, and 90%, respectively, after 1 year. The *VHL* conditional knockout mice ($VHL^{f/d}$/*Cre*) grew and developed normally up to the age of 3 months. After 3 months, $VHL^{f/d}$/*Cre* mice began to die, with 90% mortality by 1 year of age (Fig. 1*D*). The median survival time was 26 weeks. Shortly before death (1–2 days), some $VHL^{f/d}$/*Cre* mice lost weight, displayed rough fur, and a hunched body position. However, many of these mice died abruptly, with few outward symptoms.

**Multiple Hemangiomas, Angiectasis, and Angiogenesis Develop in Livers of 100% of *VHL*-conditional Knockout Mice.** The most striking phenotypic features of $VHL^{f/d}$/*Cre* mice at autopsy were observed in the liver. Surfaces of the livers were usually irregular and contained numerous dark red to black vascular lesions (Fig. 4, *A* and *B*). Examination of livers of $VHL^{f/d}$/*Cre* mice that died revealed hepatic vascular lesions containing multiple, large, thin-walled vessels filled with blood. In $VHL^{f/d}$/*Cre* mice that were euthanized at predetermined time points, the appearance of hepatic vascular lesions ranged from multiple, large, blood-filled, vascular vessels to single, small, blood-filled, vascular lesions (Fig. 4, *A* and *B*). Histological examination of the livers revealed frequent angiectasis, limited new blood vessel formation and the occurrence of multiple hemangiomas, and a single hemangiosarcoma (Fig. 4, *C–F*).

Twenty-two of 22 (100%) $VHL^{f/d}$/*Cre* mice (ages 4–12 months)

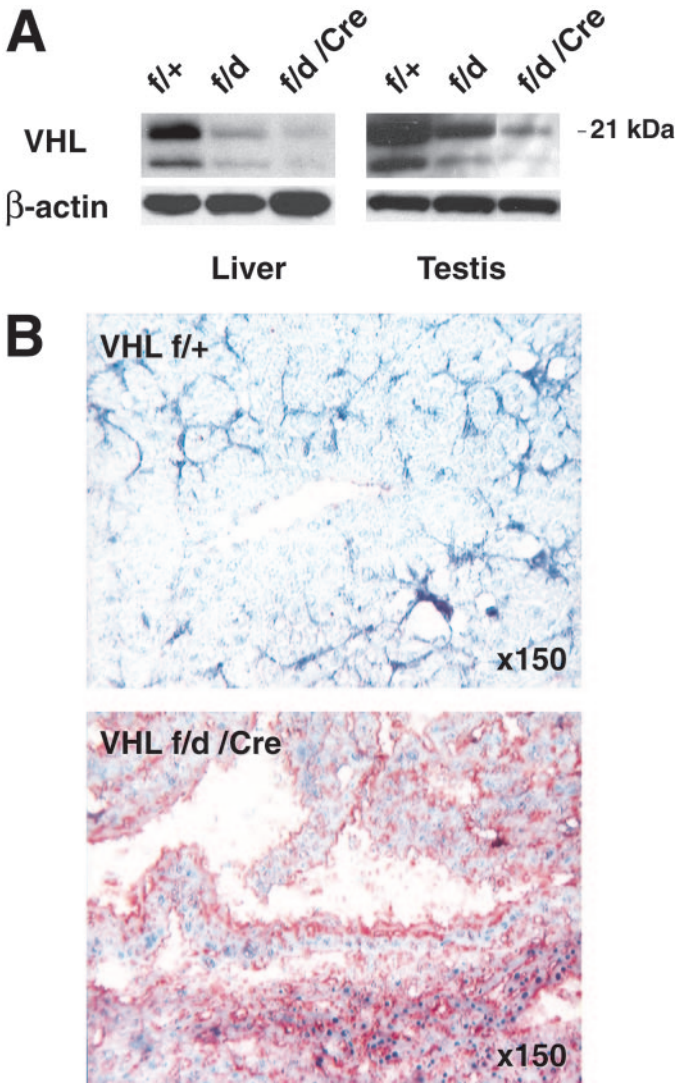


Fig. 3. Western analysis and immunohistochemistry show reduction in VHL protein levels and up-regulation of HIF-1α expression in tissues from $VHL^{f/d}$/*Cre* mice. *A*, Western analysis of immunoprecipitated pVHL extracted from livers and testes of mutant mice revealed a reduction in the long $M_r$ 21,000 and short $M_r$ 19,000 forms of pVHL in mice with one *VHL* deleted allele compared with *VHL* floxed heterozygotes. Additional reduction in pVHL levels was seen in mice with $VHL^{f/d}$/*Cre* genotype. To monitor for equal protein loading, β-actin in the cell lysate was analyzed by Western blot analysis. *B*, immunohistochemistry was performed on frozen sections from livers of $VHL^{f/d}$/*Cre* mice and $VHL^{f/+}$ control mice. HIF-1α up-regulation was seen in the endothelial cells lining the hepatic vascular lesions and surrounding regions in liver sections of $VHL^{f/d}$/*Cre* mice. No immunostaining for HIF-1α was seen in $VHL^{f/+}$ control livers. Magnification power is given.

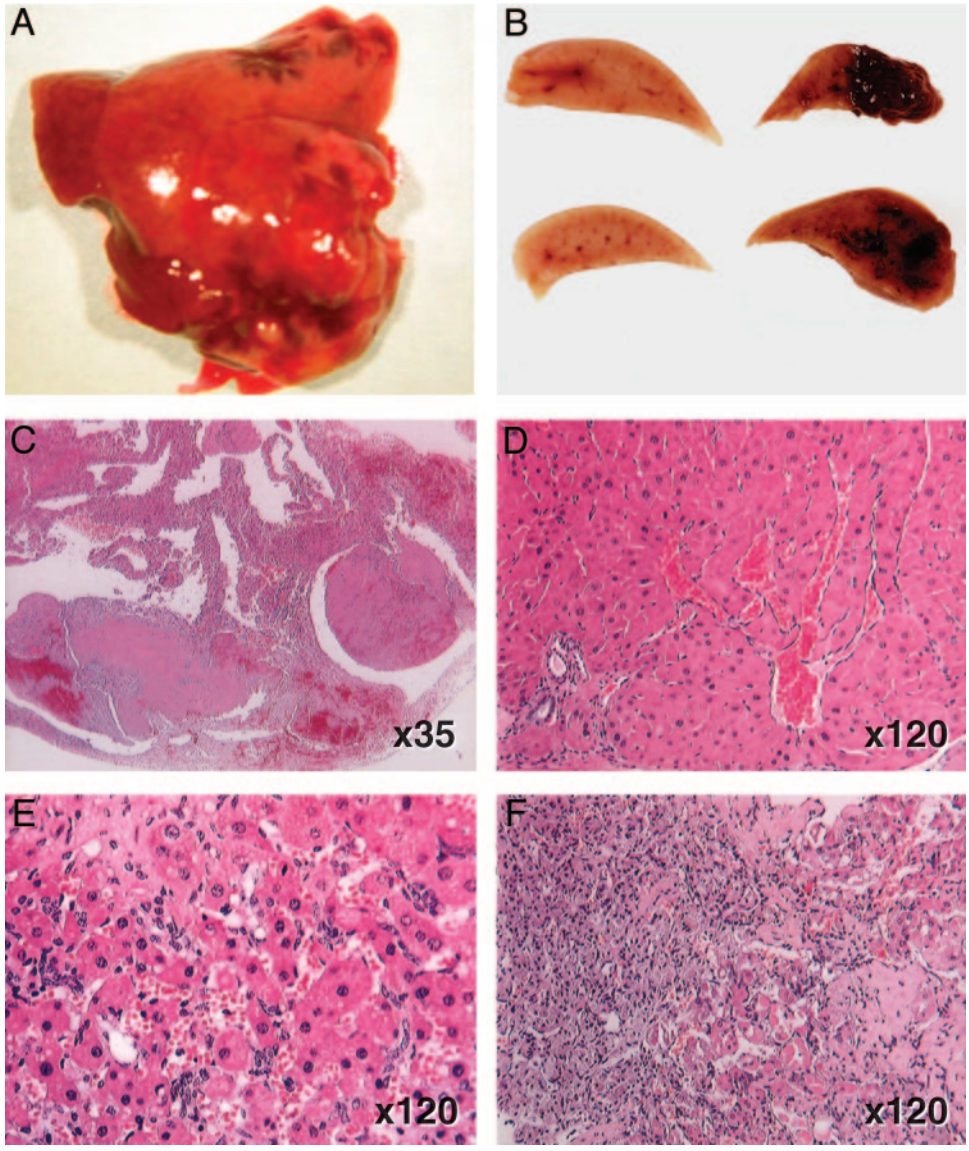


Fig. 4. Vascular phenotype in livers from mice with conditional inactivation of the *VHL* gene. *A,* on gross examination, livers from $VHL^{f/d}$/*Cre* mice had an irregular shape and rough surface with multiple, dark red zones. *B,* cut surfaces of a liver from a $VHL^{f/d}$/*Cre* mouse revealed hemangiomas in the median and left lobes (*right*), compared with the control $VHL^{f/+}$ liver (*left*). *C,* irregular vascular spaces, hemorrhage, thrombosis, and necrosis were seen on H &E staining of hepatic tissue sections at lower magnification. *D,* hyperproliferation of endothelial cells and dilated sinusoidal spaces filled with blood cells showed angiectasis in the liver. *E,* blood-filled irregular vascular spaces (hemangioma) have destroyed normal hepatic structure. *F,* a solid hemangiosarcoma was seen on H&E staining at higher magnification.

produced grossly visible hepatic hemangiomas (Table 1). For comparison, we examined age-matched control littermates and observed 4 of 22 (18%) $VHL^{f/d}$ mice with hepatic hemangiomas; these hemangiomas were smaller in size and fewer in number than those observed in $VHL^{f/d}$/*Cre* mice. Two of 22 (9%) age-matched $VHL^{f/+}$/*Cre* littermates developed hepatic hemangiomas (Table 1). The vascular liver lesions that occurred in these control $VHL^{f/d}$ and $VHL^{f/+}$/*Cre* mice presumably represent a sporadic loss of heterozygosity event, which would be predicted to inactivate the remaining wild-type *VHL* allele (one *VHL* allele is already deleted in the germ-line or by *cre* expression), predisposing the liver to develop the vascular phenotype as a result of homozygous *VHL* deletion.

Table 1 *Frequency of hepatic hemangiomas in mice with conditional inactivation of the VHL gene and control littermates*

| Genotype | Frequency of hepatic hemangiomas (no. mice with hepatic hemangioma/no. tested) |
|---|---|
| $VHL^{f/d}$/*Cre* | 22/22 (100%) |
| $VHL^{f/d}$ | 4/22 (18%) |
| $VHL^{f/+}$/*Cre* | 2/22 (9%) |
| $VHL^{f/+}$ | 0/22 (0%) |

***VHL*-conditional Knockout Mice Exhibit Defects in Spermatogenesis and Are Infertile.** As described above, in experiments with the ROSA26 reporter mice, β-actin promoter-driven Cre recombinase was strongly expressed in the testes (Fig. 2). We observed that our attempts to breed 8 $VHL^{f/d}$/*Cre* male mice with 16 C57BL/6 females over a 4-month period were unsuccessful; only 1 of 8 $VHL^{f/d}$/*Cre* male mice was able to impregnate 2 females. This led us to evaluate the male reproductive organs and spermatogenesis in male *VHL* conditional knockout mice. The testes from 16 $VHL^{f/d}$/*Cre* mice were examined at autopsy and by histopathology. We observed reduction in size and weight of the testicle, testes degeneration, and oligospermia in the testes of 16 of 16 $VHL^{f/d}$/*Cre* male mice (Fig. 5, *A* and *B*; Table 2). Sperm counts performed in 9 $VHL^{f/d}$ male mice and 9 $VHL^{f/d}$/*Cre* mice showed 2-fold ($15.19 \pm 6.45 \times 10^6$ per testis) and 30-fold ($1.13 \pm 0.95 \times 10^6$/testis; $P < 0.0001$) reduction in viable sperm, respectively, compared with sperm counts in $VHL^{f/+}$ control male animals ($33.64 \pm 4.99 \times 10^6$/testis; Table 2).

Histological examination of testes from $VHL^{f/d}$/*Cre* mice showed abnormal sperm maturation with multinucleated giant cells, few Sertoli cells, and reduced numbers of germ cells and sperm. Cross-sections of the testes showed seminiferous tubule atrophy and collapse

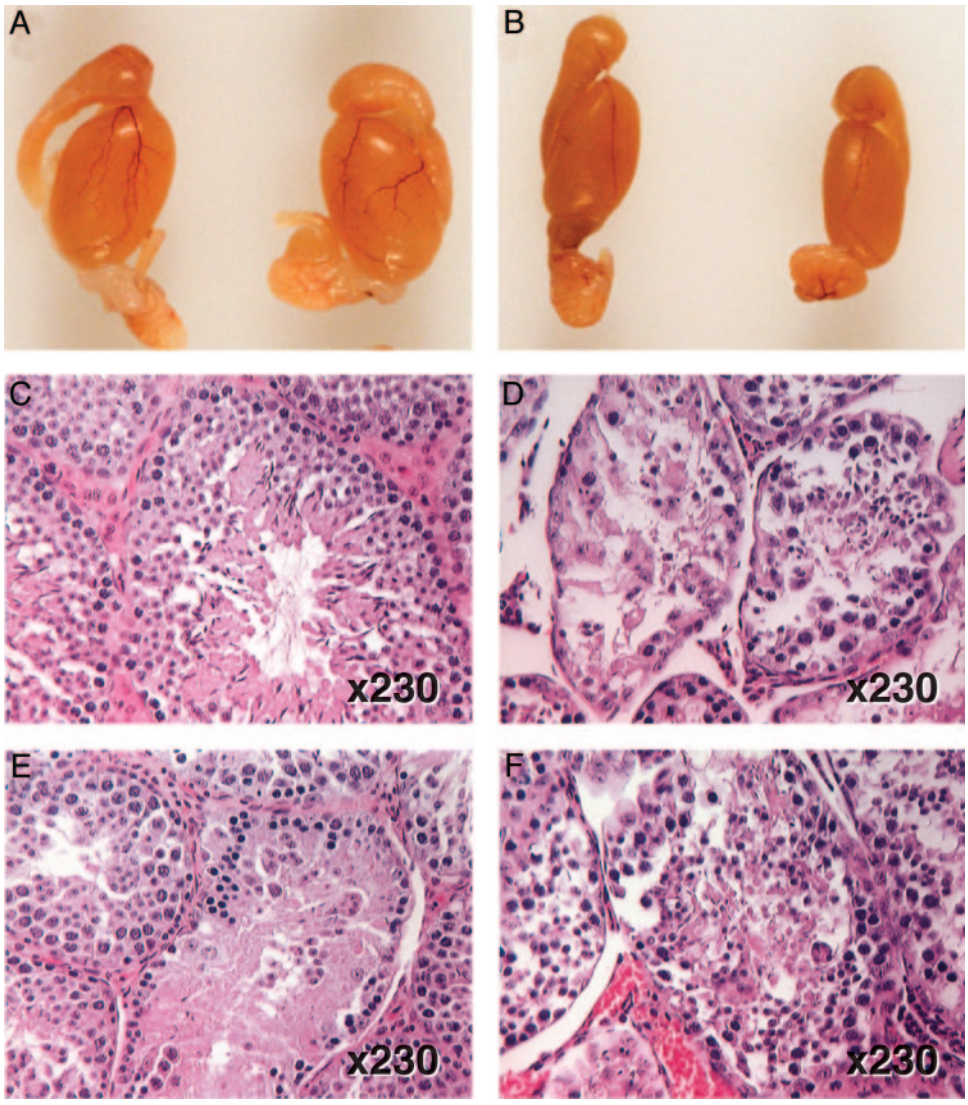


Fig. 5. Evidence of defects in spermatogenesis in testes from mice with conditional inactivation of the *VHL* gene. Testes from a 17-week-old $VHL^{f/d}$/*Cre* male are much smaller (*B*; 0.14 g) than those from a $VHL^{f/+}$ age-matched control (*A*; 0.2 g). *C,* photomicrograph of a control $VHL^{f/+}$ seminiferous tubule showing the normal structure of spermatogonia cells. *D,* cross-sections of two seminiferous tubules from $VHL^{f/d}$/*Cre* male mice display atrophy and irregular structure, with few spermatogonia cells. *E,* reduced numbers of germinal cells with almost no spermatozoa, and multinucleated giant cells, were seen in this $VHL^{f/d}$/*Cre* testis. *F,* angiectasis (dilated blood vessels) was seen in the seminiferous tubules of this $VHL^{f/d}$/*Cre* testis. Magnification power is given.

of the testicular capsule. Higher magnification of the testes displayed very few spermatozoa in the lumen of seminiferous tubules, severely reduced numbers of germinal cells, and necrotic spermatogenic cells (Fig. 5, *C–F*). Enlarged blood vessels occurred in connective tissue surrounding the seminiferous tubules. Cross-sections of the epididymal sac showed large numbers of sperm in the lumen of control mice but few sperm in the lumen of $VHL^{f/d}$/*Cre* mice (data not shown).

**Angiectasis and Angiogenesis Are Observed in the Heart, Liver, Pancreas, Lung, and Kidney of the $VHL^{f/d}$/*Cre* Mice but not in Other Organs.** In addition to the extensive vascular phenotype in the liver, new blood vessel formation (angiogenesis) in the cardiac muscle was observed in 8 of 10 $VHL^{f/d}$/*Cre* mice (Fig. 6*A*). Three of 10 kidneys from $VHL^{f/d}$/*Cre* mice displayed abnormally enlarged blood vessels (angiectasis; Fig. 6*B*). Increased numbers of blood vessels in the pancreas were observed in 6 of 10 $VHL^{f/d}$/*Cre* animals (Fig. 6*C*), and 10 of 10 animals with $VHL^{f/d}$/*Cre* genotype showed abnormally enlarged blood vessels in the liver (Fig. 6*D*).

Table 2 *Testes weight and sperm count in mice with conditional inactivation of the* VHL *gene and control littermates*

| Genotype | Testes Weight[a] (Mean ± SD) mg | Sperm Count[b] (Mean ± SD) × $10^6$ |
|---|---|---|
| $VHL^{f/d}$/*Cre* | 90 ± 30[c] | 1.13 ± 0.95[c] |
| $VHL^{f/d}$ | 200 ± 40 | 15.19 ± 6.45 |
| $VHL^{f/+}$/*Cre* | 230 ± 30 | 27.08 ± 10.08 |
| $VHL^{f/+}$ | 220 ± 30 | 33.64 ± 4.99 |

[a] Testes weight data from 14–16 male animals for each genotype group.
[b] Sperm count data from 8–9 male animals for each genotype group.
[c] $P < 0.0001$ relative to $VHL^{f/+}$ *controls; Student's t* test.

Examination of the brain, ovary, and adrenal gland revealed no vascular lesions or tumors in $VHL^{f/d}$/*Cre* mice or control littermates. No abnormalities were observed in the ovaries of any mice under 52 weeks of age. One angioma was found on the ovary of a female over 73 weeks old.

**Up-Regulated HIF1-$\alpha$ Expression Is Observed in Hepatic Vascular Lesions in $VHL^{f/d}$/*Cre* Mice.** pVHL functions to target HIF1-$\alpha$ for ubiquitin-mediated degradation during normoxic conditions. HIF1-$\alpha$ is overexpressed in the absence of functional pVHL, up-regulating hypoxia-inducible genes, which support angiogenesis. We evaluated HIF1-$\alpha$ expression by immunohistochemistry and found mosaic expression of HIF1-$\alpha$ in livers of $VHL^{f/d}$/*Cre* mice, especially in endothelial cells lining the vascular lesions, but no HIF1-$\alpha$ expression in control $VHL^{f/+}$ mice (Fig. 3*B*). In serial sections of $VHL^{f/d}$/*Cre* livers, we observed some loss of pVHL expression in a mosaic pattern (data not shown).

**Alternate Methods of Cre Recombinase Delivery to $VHL^{f/d}$ Mice Resulted in a Similar Vascular Phenotype.** As an alternative to mosaic expression of Cre recombinase driven by the $\beta$-actin pro-

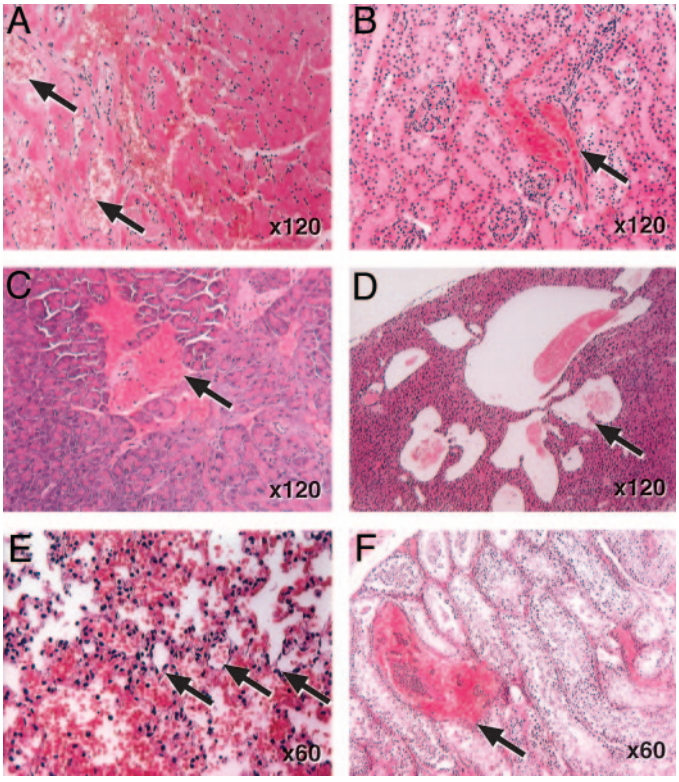


Fig. 6. Examples of the vascular phenotype showing angiectasis in multiple organs from *VHL$^{f/d}$/Cre* mice. *Arrows* indicate multiple, abnormally dilated blood vessels seen in H&E stained tissue sections of affected organs in *VHL* conditional knockout mice including (*A*) heart, (*B*) renal cortex, (*C*) pancreas, (*D*) liver, (*E*) lung (also hemorrhage), and (*F*) testis.

moter, we crossed *VHL$^{f/f}$* mice with *VHL$^{d/+}$/CreER$^{TM}$* mice carrying a transgene that produces a tamoxifen-inducible fusion protein between Cre recombinase and a mutant ligand-binding domain of the 17$\beta$-estradiol receptor (40). Most of the *VHL$^{f/d}$/CreER$^{TM}$* mice died (21 of 23) within 1 month after tamoxifen induction, with multiple hepatic hemangiomas. Only 3 of 35 *VHL$^{f/d}$* mice and 2 of 35 *VHL$^{f/+}$/CreER$^{TM}$* mice died in response to tamoxifen induction (data not shown).

We also delivered Cre recombinase to *VHL$^{f/d}$* mice and *VHL$^{f/+}$* control mice by tail vein injection of Adv/cre, a replication-defective adenovirus carrying Cre recombinase (41). At 3-, 6-, and 9-month time points, mice were euthanized and examined by autopsy. By 9 months, multiple hemangiomas had appeared in the livers of 5 of 9 Adv/cre-injected *VHL$^{f/d}$* mice but in only 2 of 6 uninjected *VHL$^{f/d}$* mice and 0 of 6 *VHL$^{f/+}$* Adv/cre-injected mice had developed a vascular phenotype (data not shown).

**Modifier Genes Present in Different Mouse Strains May Enhance the Phenotype of the *VHL*-conditional Knockout Mice.** Reports in the literature suggest that mouse models for human disease may develop variable phenotypes, which are strain-dependent. It has been proposed that these differences in phenotype are because of the presence of polymorphic variants in certain modifier genes in some strains, which may contribute to the development of the disease phenotype. In this report we describe a *VHL* conditional knockout mouse model produced in a C57BL/6 background, which developed liver hemangiomas in 100% (22 of 22) of animals by 1 year. Only 18% (4 of 22) of *VHL$^{f/d}$* control littermates developed liver hemangiomas during that period. We evaluated the effect of different strains (modifier genes) on phenotype development by introducing the *VHL* deleted allele into BALB/c and A/J mouse strains, and observed first generation offspring for phenotypic changes. After 18 months, 88% (7 of 8) of *VHL$^{d/+}$*BALB/c mice developed liver hemangiomas, and 67% (4 of 6) of *VHL$^{d/+}$* A/J mice developed hepatic vascular lesions, representing a substantial increase in the number of vascular lesions when compared with C57BL/6 *VHL$^{f/d}$* mice.

## DISCUSSION

Development of animal models for human cancer syndromes provides an *in vivo* system with which to dissect and study the sequence of events leading from preneoplastic changes to tumor initiation and progression. In addition, an *in vivo* cancer model will provide an important research tool for testing new cancer treatments. Using Cre/lox site-specific recombination, we have developed a *VHL* conditional knockout mouse model for VHL disease, which mimics this vascular-rich tumor syndrome in humans. Embryonic lethality has been partially avoided through the use of mosaic Cre expression. The *VHL* mouse model described in this report, with widespread stochastic expression of Cre recombinase, developed a more extensive vascular phenotype than observed in a previously reported *VHL* conditional knockout mouse model (34), including hepatic hemangiomas, and neovascularization and enlarged blood vessels (angiectasis) in a number of organs, notably the *VHL* target organs pancreas and kidney. The regulation of angiogenic pathways by pVHL through oxygen-dependent ubiquitin-mediated degradation of HIF1-$\alpha$ is disrupted by conditional inactivation of the *VHL* gene in our mouse model. Single layers of endothelial cells, which line the multiple, dilated blood vessels, were seen as sites of HIF1-$\alpha$ up-regulation resulting in increased angiogenic signaling and a multiorgan vascular phenotype. In mice, unlike humans, the vascular phenotype was most extensive in the liver, resulting in the development of hemangiomas and hemangiosarcomas in 100% of *VHL* conditional knockout mice.

Here we present important new findings not reported previously in *VHL* knockout mouse models, which suggest a novel role for pVHL in mouse spermatogenesis. Oligospermia, reduction in testicular weight, and infertility were observed in male *VHL$^{f/d}$/Cre* mice. Histological evaluation of the testes suggests that loss of pVHL function may interfere with maturation of spermatogonia cells to mature sperm. *VHL* expression was shown to be high in developing seminiferous tubules of the mouse testis at embryonic days 10–13, but low in the ovary (44). An oxygen-dependent isoform of HIF (HIF1-$\alpha$1.2) is expressed in testis (45, 46), permitting one to speculate that impaired regulation of HIF may result in defects in spermatogenesis.

Evidence is accumulating for a role of *VHL* in induction of cell differentiation and growth arrest through integration of cell-cell and cell-extracellular matrix signals. In the absence of pVHL, renal cells do not differentiate, and the cell cycle proceeds (47). In addition, pVHL was shown to play a role in neuronal differentiation (48, 49). Inactivation of pVHL may also inhibit differentiation in the testis, resulting in defects in spermatogenesis. Inability to exit the cell cycle, a "gatekeeper" function of pVHL, may induce apoptotic cell death.

The VHL protein was shown recently to bind to and stabilize microtubules (30). The testicular degeneration seen in the male *VHL$^{f/d}$/Cre* mice may be a consequence of loss of microtubule stability within the testis, which is important in mitosis as well as meiosis. MutS homologue 4 mouse knockouts, defective in postreplicative DNA mismatch repair and meiotic recombination, display testicular degeneration and are infertile (50). Msh4, which is expressed predominantly in the testis, associates with VBP1 (51) *in vivo* and *in vitro*. Disruption of the *VBP1* homologue in yeast (*GIM2/PAC10*) has been shown to interfere with $\alpha$-tubulin biogenesis; the lethal phenotype can be rescued by mammalian VBP1 (52), identified as a VHL binding partner in a yeast-two hybrid system (53). Additional studies will be

required to ascertain the mechanism by which absence of pVHL leads to defective spermatogenesis.

The *VHL* conditional knockout mouse model described in this report is the second attempt to create an animal model for the VHL disease using conditionally targeted *VHL* alleles. Haase *et al.* (34) used albumin promoter driven-Cre expression previously to target *VHL* inactivation in the liver, which produced enlarged blood vessels and premature death. In our mouse model, $\beta$-actin promoter-driven mosaic expression of Cre recombinase, resulting in homozygous inactivation of *VHL* in certain cells within target organs, more closely mimics the human disease condition in which loss of the wild-type *VHL* allele occurs in cells carrying a germ-line V*HL* mutation. Stochastic *cre* expression produced not only an extensive vascular phenotype in the liver, but also angiectasis and angiogenesis in the pancreas, kidney, spleen, and heart, and defective spermatogenesis, reflecting a novel role for pVHL.

Different modifier gene alleles present in the different mouse genetic backgrounds may enhance or alter phenotype development in mouse models of human cancer. Strain differences were shown to contribute to the disease phenotype in a mouse model of hereditary hemorrhagic telangiectasis (54) and in a mouse model for tuberous sclerosis (55, 56). The differences in phenotypic consequence of the heterozygous *VHL* deleted allele, which we observed among three different mouse strains in our study, suggest that strain-specific modifier genes may provide protection against the formation of hepatic hemangiomas in $VHL^{f/d}$ C57BL/6 mice, or may enhance the development of the vascular phenotype in $VHL^{d/+}$ BALB/c or A/J mice. Identification of these modifier genes may provide insight into the events leading to the development of the vascular phenotype and suggest additional angiogenic effectors to knockout in our *VHL* mouse model.

Multiple hepatic hemangiomas produced by conditional inactivation of the *VHL* gene resulted in premature death, and the lethality of this phenotype prevented long-term observation of mice for possible changes in the kidneys or CNS. Delivery of Cre recombinase either by i.v. injection with adenovirus carrying Cre recombinase, or tamoxifen induction of CreER$^{TM}$ recombinase resulted in the formation of hepatic hemangiomas and increased mortality. Organ-specific (*i.e.* kidney or CNS) delivery of Cre recombinase will be required to circumvent the lethality of the hepatic vascular phenotype produced by inactivation of the *VHL* gene in mice.

In summary, we have generated a *VHL* conditional knockout mouse model with an extensive vascular phenotype in multiple organs, and infertility and reduced sperm count in males, which will prove useful for testing antiangiogenic drug treatments, as well as understanding the novel role for *VHL* in spermatogenesis. Renal cell carcinomas, pheochromocytomas, and CNS hemangioblastomas, which are observed in patients affected with VHL, were not observed in the *VHL* conditional knockout mouse model described in this report or by Haase *et al.* (34). This observation raises the question of whether other events, perhaps inactivation of other modifier genes, may be essential to the development of these tumor phenotypes. Breeding experiments introducing null alleles of modifier genes (*i.e.* tumor suppressor genes) into the *VHL* conditional knockout mouse background are under way to test this hypothesis. Cre expression and targeted deletion of a *VHL* floxed allele in renal cells await the development of a kidney-specific *Cre* transgenic animal.

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