Hepatic Vascular Tumors, Angiectasis in Multiple Organs, and Impaired Spermatogenesis in Mice with Conditional Inactivation of the VHL Gene

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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. Homozygotic 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. VHLlox/Cre mice developed multiple, hepatic hemangiomas that led to premature death, as well as angiectasis and angiogenesis in multiple organs. Interestingly, testes of male VHLlox/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 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 concurrent 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 HIFs, may participate in tumorogenesis. 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 tumorogenesis. 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 microscopically focci 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-
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 EcoRI site upstream of exon 2 for G418 selection, and a second loxP site was inserted in a unique HindIII site, 5.8 kb downstream, destroying the HindIII 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 NotI 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. HindIII-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 HindIII fragment for the VHL wild-type allele, an 18.2 kb HindIII fragment for the VHL floxed allele (without neo gene), and a 12.4 kb HindIII 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 were male and female (41) and bred to generate a human VHL f/f mouse. To generate a conditional knockout mouse, we crossed VHL−/+/CreERTM mice with VHL+/CreERTM mice and selected for VHL−/CreERTM offspring by Southern blot analysis of HindIII-digested tail DNA.

**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% CO2 atmosphere for 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 UltraVision Mouse Tissue Detection System (NeoMarkers).

**Western Analysis.** Mice were killed by CO2 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 Na3VO4, 0.5 mM dithiothreitol, 10 μg/ml leupeptin 10 μg/ml, 1 mM phenylephylsulfonfluoride, 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% PBS buffer (pH 7.4). After incubation with antiserum VHL M-20 antibody (1:400; Santa Cruz Biotechnology), the membrane was washed with PBS for 5 min and then incubated with horseradish peroxidase-conjugated secondary antibody (Amer sham Pharmacia Biotech). Signals were detected using the enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech). Measurements were taken in millimeter 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 mice, we constructed a VHL targeting vector, in which exons 2 and 3 of the mouse VHL gene were floxed (Fig. 1A). 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 was 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.

**Infertility Testing.** Eight 10–15-week-old VHL+/CreERTM males were bred with 16 age-matched C57BL/6 females over a 4-month 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% CO2 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).
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 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. 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^+/flox) mice with β-actin cre transgenic
mice produced with a heterozygous VHL deleted allele (VHLf/d/Cre) when Cre expression occurred in the germ cells and VHLf/d/Cre mice, when Cre expression did not occur in the germ cells, but instead mosaically in developing tissues. Crosses between VHL homozygous floxed (VHLf/f) mice and VHLf/d/Cre mice produced VHLf+/d/C+, VHLf+f/C+, VHLf+f/d, and VHLf+/d/f mice (Vhl conditional knockout). Some VHLf/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 HindIII-digested tail DNA (Fig. 1, A and B).

Comparison of growth curves for VHLf+/f mice, VHLf/f mice, VHLf/d+/Cre mice, and VHLf/d/f 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. 1C).

VHL Protein Levels Were Reduced in Tissues of Mice with VHL Inactivation. To compare pVHL expression in VHL heterozygous (VHLf/f) and homozygous deleted mice (VHLf/f/Cre) with expression in VHL heterozygous floxed mice (VHLf/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, 21,000 and M, 19,000, respectively) were identified in liver and testis tissues. Reduced expression levels of VHL protein were detected in VHLf/f mice compared with VHLf+/f mice (Fig. 3A). As a result of mosaic tissue expression of Cre recombinase, we detected an additional reduction in pVHL expression in VHLf/f 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 VHLf/f, VHLf/f+, and VHLf/d+/Cre control littermates grew and developed normally with survival rates of 100%, 100%, and 90%, respectively, after 1 year. The VHL conditional knockout mice (VHLf/d/Cre) grew and developed normally up to the age of 3 months. After 3 months, VHLf/d/Cre mice began to die, with 90% mortality by 1 year of age (Fig. 1D). The median survival time was 26 weeks. Shortly before death (1–2 days), some VHLf/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 VHLf/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 VHLf/d/Cre mice that died revealed hepatic vascular lesions containing multiple, large, thin-walled vessels filled with blood. In VHLf/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%) VHLf/d/Cre mice (ages 4–12 months) died. At autopsy, livers were filled with blood. In VHLf/d/Cre mice that died revealed hepatic vascular lesions containing multiple, large, thin-walled vessels filled with blood. In VHLf/d/Cre mice that were euthanized at predetermined time points, the appearance of hepatic vascular lesions tended to range 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).

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produced grossly visible hepatic hemangiomas (Table 1). For comparison, we examined age-matched control littermates and observed 4 of 22 (18%) \textit{VHL}^{f/d} mice with hepatic hemangiomas; these hemangiomas were smaller in size and fewer in number than those observed in \textit{VHL}^{f/d/Cre} mice. Two of 22 (9%) age-matched \textit{VHL}^{f/d/H11001}/Cre littermates developed hepatic hemangiomas (Table 1). The vascular liver lesions that occurred in these control \textit{VHL}^{f/d} and \textit{VHL}^{f/d/H11001}/Cre mice presumably represent a sporadic loss of heterozygosity event, which would be predicted to inactivate the remaining wild-type \textit{VHL} allele (one \textit{VHL} allele is already deleted in the germ-line or by \textit{cre} expression), predisposing the liver to develop the vascular phenotype as a result of homozygous \textit{VHL} deletion.

\textbf{Table 1} \textit{Frequency of hepatic hemangiomas in mice with conditional inactivation of the VHL gene and control littermates}

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Frequency of hepatic hemangiomas (no. mice with hepatic hemangioma/no. tested)</th>
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</thead>
<tbody>
<tr>
<td>\textit{VHL}^{f/d/Cre}</td>
<td>22/22 (100%)</td>
</tr>
<tr>
<td>\textit{VHL}^{f/d}</td>
<td>4/22 (18%)</td>
</tr>
<tr>
<td>\textit{VHL}^{f/d/H11001}/Cre</td>
<td>2/22 (9%)</td>
</tr>
<tr>
<td>\textit{VHL}^{f/d/H11001}</td>
<td>0/22 (0%)</td>
</tr>
</tbody>
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\textit{VHL}-conditional Knockout Mice Exhibit Defects in Spermatogenesis and Are Infertile. As described above, in experiments with the ROSA26 reporter mice, \beta-actin promoter-driven Cre recombinase was strongly expressed in the testes (Fig. 2). We observed that our attempts to breed 8 \textit{VHL}^{f/d/Cre} male mice with 16 C57BL/6 females over a 4-month period were unsuccessful; only 1 of 8 \textit{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 \textit{VHL} conditional knockout mice. The testes from 16 \textit{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 \textit{VHL}^{f/d/Cre} male mice (Fig. 5, A and B; Table 2). Sperm counts performed in 9 \textit{VHL}^{f/d} male mice and 9 \textit{VHL}^{f/d/Cre} mice showed 2-fold (15.19 ± 6.45 × 10^6 per testis) and 30-fold (1.13 ± 0.95 × 10^9/testis; \textit{P} < 0.0001) reduction in viable sperm, respectively, compared with sperm counts in \textit{VHL}^{f/d/H11001} control male animals (33.64 ± 4.99 × 10^9/testis; Table 2).

Histological examination of testes from \textit{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.
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 control mice but few sperm in the lumen of VHLf/d/Cre mice (data not shown).

Angiectasis and Angiogenesis Are Observed in the Heart, Liver, Pancreas, Lung, and Kidney of the VHLf/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 VHLf/d/Cre mice (Fig. 6A). Three of 10 kidneys from VHLf/d/Cre mice displayed abnormally enlarged blood vessels (angiectasis; Fig. 6B). Increased numbers of blood vessels in the pancreas were observed in 6 of 10 VHLf/d/Cre animals (Fig. 6C), and 10 of 10 animals with VHLf/d/Cre genotype showed abnormally enlarged blood vessels in the liver (Fig. 6D).

Examination of the brain, ovary, and adrenal gland revealed no vascular lesions or tumors in VHLf/d/Cre mice or control littersmates. 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-α Expression Is Observed in Hepatic Vascular Lesions in VHLf/d/Cre Mice. pVHL functions to target HIF1-α for ubiquitin-mediated degradation during normoxic conditions. HIF1-α is overexpressed in the absence of functional pVHL, up-regulating hypoxia-inducible genes, which support angiogenesis. We evaluated HIF1-α expression by immunohistochemistry and found mosaic expression of HIF1-α in livers of VHLf/d/Cre mice, especially in endothelial cells lining the vascular lesions, but no HIF1-α expression in control VHLf/+ mice (Fig. 3B). In serial sections of VHLf/d/Cre livers, we observed some loss of pVHL expression in a mosaic pattern (data not shown).

Alternate Methods of Cre Recombinase Delivery to VHLf/d Mice Resulted in a Similar Vascular Phenotype. As an alternative to mosaic expression of Cre recombinase driven by the β-actin pro-

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

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Testes Weighta (Mean ± SD) mg</th>
<th>Sperm Countb (Mean ± SD) × 10⁶</th>
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<tbody>
<tr>
<td>VHLf/d/Cre</td>
<td>90 ± 30</td>
<td>1.13 ± 0.95</td>
</tr>
<tr>
<td>VHLf/d/Cre</td>
<td>200 ± 40</td>
<td>15.19 ± 6.45</td>
</tr>
<tr>
<td>VHLf/d/Cre</td>
<td>230 ± 30</td>
<td>27.08 ± 10.08</td>
</tr>
<tr>
<td>VHLf/d/Cre</td>
<td>220 ± 30</td>
<td>33.64 ± 4.99</td>
</tr>
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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.

P <0.0001 relative to VHLf/d/+ controls; Student’s t test.
VASCULAR LESIONS AND SPERM DEFECTS IN VHL KO MICE

moter, we crossed VHLf/f mice with VHLd/d/CreERTM mice carrying a transgene that produces a tamoxifen-inducible fusion protein between Cre recombinase and a mutant ligand-binding domain of the 17β-estradiol receptor (40). Most of the VHLf/f/CreERTM mice died (21 of 23) within 1 month after tamoxifen induction, with multiple hepatic hemangiomas. Only 3 of 35 VHLf/d mice and 2 of 35 VHLf/d/CreERTM mice died in response to tamoxifen induction (data not shown).

We also delivered Cre recombinase to VHLf/d mice and VHLf/d 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 VHLf/d mice but in only 2 of 6 uninjected VHLf/d mice and 0 of 6 VHLf/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 VHLf/d control littersmates 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 VHLf/f BALB/c mice developed liver hemangiomas, and 67% (4 of 6) of VHLf/d A/J mice developed hepatic vascular lesions, representing a substantial increase in the number of vascular lesions when compared with C57BL/6 VHLf/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-α 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-α 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 VHLf/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-α1.2) is expressed in testsis (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 VHLf/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 testsis, associates with VBP1 (51) in vivo and in vitro. Disruption of the VBP1 homolog in yeast (GIM2/PAC10) has been shown to interfere with α-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, β-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 VHL 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 telangiectasia (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+/− C57BL/6 mice, or may enhance the development of the vascular phenotype in VHL+/− 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 CreERTM recombinase resulted in the formation of hepatic hemangiomas and increased mortality. Organ-specific (for testing antiangiogenic drug treatments, as well as understanding pheochromocytomas, and CNS hemangioblastomas, which are ob-

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