SHORT COMMUNICATION

High Frequency of Ovarian Cyst Development in *Vhl*^{2B/+}; *Snf5*^{+/-} Mice

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The new paradigm of mutations in chromatin-modifying genes as driver events in the development of cancers has proved challenging to resolve the complex influences over disease phenotypes. In particular, impaired activities of members of the SWI/SNF chromatin remodeling complex have appeared in an increasing variety of tumors. Mutations in *SNF5*, a member of this ubiquitously expressed complex, arise in almost all cases of malignant rhabdoid tumor in the absence of additional genetic alterations. Therefore, we studied how activation of additional oncogenic pathways might shift the phenotype of disease driven by *SNF5* loss. With the use of a genetically engineered mouse model, we examined the effects of a hypomorphic *Vhl*^{2B} allele on disease phenotype, with a modest up-regulation of the hypoxia response pathway. *Snf5*^{+/-}; *Vhl*^{2B/+} mice did not demonstrate a substantial difference in overall survival or a change in malignant rhabdoid tumor development. However, a high percentage of female mice showed complex hemorrhagic ovarian cysts, a phenotype rarely found in either parental mouse strain. These lesions also showed mosaic expression of *SNF5* by immunohistochemistry. Therefore, our studies implicate that modest changes in angiogenic regulation interact with perturbations of SWI/SNF complex activity to modulate disease phenotypes. (*Am J Pathol* 2018, 188: 1510–1516; <https://doi.org/10.1016/j.ajpath.2018.03.010>)

SWI/SNF complex members have been recognized as mutation targets for cancer for many years, but only recently have they been recognized as commonly mutated across a wide range of tumors.^{1,2} However, little information exists about what host or tissue factors influence the range of tumor susceptibilities associated with individual members of the SWI/SNF complexes. For example, malignant rhabdoid tumors (MRTs) are a rare and aggressive pediatric malignancy associated with a poor prognosis. Most MRTs possess mutations or deletions that inactivate the *SNF5/INI1/SMARCB1* tumor suppressor, a member of the SWI/SNF chromatin remodeling complex.³ Recent next-generation sequencing studies of MRTs have found that the tumors generally possess only *SNF5* mutations, in contrast to most adult malignancies, which typically harbor >50 genetic events.³ This observation implicates alterations in global nucleosome positioning, observed on *SNF5* loss,

and the concomitant changes in gene expression and gene enhancers as the primary drivers of tumorigenesis.^{4,5} However, how the tumor microenvironment may influence the development of tumors driven by *SNF5* or other SWI/SNF complex mutation remains unclear.

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Table 1 Characteristics of *Snf5*^{-/+}, *Vhl*^{2B/+}, and *Snf5*^{-/+};*Vhl*^{2B/+} Mice

Genotype	Tumor histology	Tumors or cysts/total mice, <i>n</i>	Mean age, months	Total penetrance (mice with tumors or cysts/total mice, <i>n</i>)
<i>Vhl</i> ^{2B/+} ; <i>Snf5</i> ^{-/+}	Malignant rhabdoid tumor	1/28	17.5	43% (12/28)*
	Ovarian cyst	8/15 (females only)	20.5	
	Lymphoma	3/28	20	
	Choroid plexus carcinoma	1/28	15	
	Fibrosarcoma	1/28	16	
<i>Vhl</i> ^{2B/+}	Hepatocellular carcinoma	1/26	15	13% (3/24)
	Ovarian cyst	1/6 (females only)	17	
	Lymphoma	1/26	19	
<i>Snf5</i> ^{-/+}	Malignant rhabdoid tumor	3/23	15	17% (4/23)
	Lymphoma	1/23	22	

*Three mice had two tumors.

Aggressive human tumor growth usually relies on several important properties, including the ability to invade normal tissues, escape immune surveillance, and generate new vasculature.⁶ Neovascularization ensures an adequate blood supply for actively growing tumors often through activation of the hypoxia response signaling pathway, mediated by the family of hypoxia inducible factor transcription factors, hypoxia inducible factor (HIF)1 and HIF2.⁷ Whether a state of permissive neovascularization can contribute to the disease spectrum associated with SNF5 loss remains unclear. Because one report found a relation between SWI/SNF complex activity and hypoxia signaling, the potential link between SNF5 loss and angiogenesis warrants further investigation.^{8,9}

To determine whether factors that enhance angiogenic signaling influence MRT progression after SNF5 inactivation, two genetically engineered mouse models were used. *Snf5*^{+/-} heterozygous mice develop tumors that closely resemble human MRTs in approximately 10% to 30% of animals after a long latency (>8 months).¹⁰ *Vhl*^{2B/+} mice carry a missense mutation in exon 3, resulting in a hypomorphic allele displaying reduced, but detectable, protein stability and expression. Mice are phenotypically normal with the exception of cyst development in the kidney and adrenal glands.¹¹ However, tissue analysis indicates a modest up-regulation of the hypoxia response pathway with elevated expression of HIF target genes involved in angiogenesis.¹¹ Therefore, this model was selected to promote a modest enhancement in the hypoxia response pathway through a heterozygous mutation, mimicking the kind of mild variants that might contribute to MRT development, or development of other tumors related to *Snf5* deletion. *Snf5*^{+/-};*Vhl*^{2B/+} mice were generated to address whether increased angiogenic signaling influenced tumor development in an *in vivo* model system. Our results indicate that the heterozygous mutation in *Vhl* did not alter MRT penetrance or latency. However, a dramatic increase was observed in the number of hemorrhagic ovarian cysts found in female mice. These results further associate perturbations in SWI/SNF complex components with abnormalities in the ovaries of genetically engineered mouse models.^{12–19}

Materials and Methods

Generation of *Snf5*^{+/-};*Vhl*^{2B/+} Mice

The generation, screening, and characterization of *Vhl*^{2B/+} transgenic and *Snf5*^{+/-} mice have been described previously.^{11,20} *Vhl*^{2B/+} transgenic were maintained on a mixed C57BL/6 and BalbC background. The *Snf5*^{+/-} mice were backcrossed to C57BL/6 mice for at least nine generations to produce congenic *Snf5*^{+/-} mice.²¹ *Snf5*^{+/-};*Vhl*^{2B/+} mice were derived by crossing *Vhl*^{2B/+} mice with *Snf5*^{+/-} mice and using only F1 generation mice. Mice with resulting genotypes, *Vhl*^{2B/+}, *Snf5*^{+/-}, and *Vhl*^{2B/+};*Snf5*^{+/-} were born with the expected Mendelian frequencies. All procedures were approved by the University of North Carolina Institutional Animal Care and Use Committees.

Genotyping

Mice were genotyped by PCR amplification of genomic DNA from either mouse tails or cell lines. Mouse genomic DNA was extracted by incubating tissues with buffer A (0.2 mmol/L EDTA pH 8.0 and 25 mmol/L NaOH) at 95°C for 1 hour. The lysate was neutralized by the same volume of buffer B (40 mmol/L Tris-HCl), followed by vigorous mixing with a vortex mixer. Debris were pelleted, and the supernatant was used in PCR amplification by using the EasyStart 50 PCR Kit (Molecular Bio-Products Inc., San Diego, CA). The wild-type and null *Snf5* alleles and the *Vhl*^{2B} allele were detected as previously reported.^{11,22}

Histopathology and ICH

All tumors identified in this study were fixed in 10% neutral-buffered formalin for 16 to 20 hours, washed in running water for 3 minutes, and saved in 70% ethanol. Tissues were routinely processed and embedded, and 5-μm sections were stained with hematoxylin and eosin or processed for immunohistochemistry (IHC) as previously described.^{21,22} For IHC, antigen retrieval was performed

Figure 1 Histopathology and SNF5 immunohistochemistry (IHC) of tumors from *Snf5*^{+/-} and *Snf5*^{+/-};*Vhl*^{2B/+} mice. **A** and **B**: Representative rhabdoid tumors from the *Snf5*^{+/-} mice were stained for histology by hematoxylin and eosin (H&E) (**A**) and for expression of SNF5 by IHC (**B**). The **red arrows** denote SNF5-positive infiltrating normal lymphocytes and capillary endothelial cells that serve as positive internal controls.²¹ **C** and **D**: The choroid plexus carcinoma (CA) from the *Snf5*^{+/-};*Vhl*^{2B/+} mice were stained for histology by H&E (**C**) and for expression of SNF5 by IHC (**D**). Varying levels of SNF5 expression are found in >99% of the cells. Original magnification, ×400.

with 1.0 mmol/L EDTA, pH 8.0 (Zymed, South San Francisco, CA) in a steam pressure cooker at 125°C for 30 seconds, followed by a cool down set point at 90°C for 10 seconds (Decloaking Chamber; BioCare Medical, Walnut Creek, CA) according to the manufacturer's instructions, followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were pretreated with Peroxidase Block (Dako USA, Carpinteria, CA) for 5 minutes to quench endogenous peroxidase activity. For SNF5, monoclonal mouse anti-SNF5 antibody (BD Biosciences, San Jose, CA; clone 25/BAF47, catalog number 612111) was applied at dilution 1:100 in Dako diluent for 1 hour. For ERG, rabbit polyclonal anti-ERG antibody [Abcam EPR3864(2)] was applied at dilution 1:2000 in Dako diluent for 1 hour. Slides were washed in 50 mmol/L Tris-HCl, pH 7.4, and detected with anti-mouse Envision + kit (Dako) according to the manufacturer's instructions. After further washing, immunoperoxidase staining was developed by using a diaminobenzidine chromogen (Dako) and counterstained with hematoxylin. Hematoxylin and eosin- and IHC-stained sections were evaluated independently by experienced surgical pathologists (C.D.F., A.N.K., and D.G.H.) and a veterinary pathologist (A.B.R.) in blinded fashion.^{16,20,23}

Results

Snf5^{+/-};*Vhl*^{2B/+} Mice Do Not Show Changes in MRT Penetrance or Latency

To assess potential interactions between SNF5 and VHL inactivation, genetically engineered mouse models that possessed a null allele of *Snf5* and a mutant allele of *Vhl* were generated. Although *Snf5*^{+/-} mice developed

normally, facial and nervous system tumors that closely resemble human MRT and lymphomas appeared after a long latency period.²⁰ *Vhl*^{2B/+} mice also showed normal development with a phenotype characterized by vascular abnormalities in the kidney, and in mice receiving treatment with ethylnitrosourea, tumor phenotypes ranged from hyperplasia to adenocarcinoma.¹¹ *Snf5*^{+/-}, *Vhl*^{2B/+}, and *Snf5*^{+/-};*Vhl*^{2B/+} mice were generated and followed for tumor development or other phenotypes for up to 24 months. The typical incidence of MRTs (3 of 23; 13%), as well as one mouse with lymphoma, in the *Snf5*^{+/-} mice were observed (Table 1). The average latency (15 months) was similar to a previous report from our laboratory.²² As expected, no evidence of MRT development was observed in the *Vhl*^{2B/+} mice (Table 1). A substantial difference in the penetrance of the MRTs between *Snf5*^{+/-} mice and *Snf5*^{+/-};*Vhl*^{2B/+} mice was not observed (Table 1). Although the incidence of MRTs in the *Snf5*^{+/-};*Vhl*^{2B/+} mice appeared lower (1 of 28; 4%), the development of other tumors combined with the long latency of the MRTs may have precluded determination of the MRT end point in these mice. Each group developed facial or brain MRTs, in agreement with previous reports; however, one *Snf5*^{+/-};*Vhl*^{2B/+} mouse developed both a facial MRT and a lymphoma.²⁰⁻²² The two tumor phenotypes were confirmed independently by two pathologists. Therefore, no evidence of cooperativity of *Vhl* mutation and *Snf5* loss in MRT development was found.

MRTs appearing in *Snf5*^{+/-} mice lose expression of the remaining wild-type *Snf5* allele.²⁰⁻²² Therefore, SNF5 protein expression was examined in tumors in mice by IHC. In a typical MRT, a complete loss of SNF5 expression was observed in MRTs from *Snf5*^{+/-} mice (Figure 1, A and B). In contrast, a choroid plexus carcinoma from a *Snf5*^{+/-};*Vhl*^{2B/+} mouse retained SNF5 expression (Figure 1, C and D).

Figure 2 SNF5 expression in ovaries from *Snf5*^{+/-};*Vhl*^{2B/+} and *Vhl*^{2B/+} mice. **A** and **B**: Papillary endothelial hyperplasia (PEH; **yellow outline**) and vascular sinuses (VS) resembling a cavernous hemangioma surrounding a large ovarian thrombus (T) in a *Snf5*^{+/-};*Vhl*^{2B/+} mouse. **C**: SNF5 expression by immunohistochemistry (IHC) showing endothelial cells and stromal cells (S) from cyst in **A** and **B**. The **inset** shows ERG staining of endothelial cells lining the papillary tufts. **D–F**: The single *Vhl*^{2B/+} mouse with ovarian pathology. **E**: Represents the area marked by the **black box** in panel **D**. **F**: SNF5 IHC from the area marked by the **black box** in panel **D**. Intact SNF5 expression in cells lining hemorrhagic cysts are denoted by the **arrowheads**. **G**: Short IHC development time. **H**: Long IHC development time demonstrating variable levels of SNF5 expression by IHC in normal mouse ovary. **Stars** denote interfollicular and subepithelial stromal cells that lack SNF5 by IHC; **asterisks**, hemosiderin-laden macrophages. Original magnification: $\times 40$ (**A** and **D**); $\times 100$ (**B**); $\times 200$ (**C**, **inset**, and **E**); $\times 400$ (**C** and **F–H**). B, ovarian bursa (marked by **black arrow**); CF, cystic follicle; GC, granulosa cells of a small follicle in the remaining ovarian cortex (**yellow circles**); H, hemorrhage; HF, hemorrhagic follicle (**black arrow**); OSE, ovarian surface epithelium; Ovi, oviduct.

Snf5^{+/-};*Vhl*^{2B/+} Female Mice Develop Hemorrhagic Ovaries

Although a significant change in the incidence of MRTs in the *Snf5*^{+/-};*Vhl*^{2B/+} mice was not observed, large ovarian masses were found in $>50\%$ of the *Snf5*^{+/-};*Vhl*^{2B/+} female mice (8 of 15; 53%; $P < 0.0001$, using χ^2 analysis) (Table 1). The ovarian masses appeared to consist of poorly vascularized, hemorrhagic cysts (Figure 2, A and B). Papillary endothelial hyperplasia was observed in *Snf5*^{+/-};*Vhl*^{2B/+} female mice at the interface between the normal parenchyma and organized thrombi, surrounded by a network of irregular, dilated, blood-filled, endothelial-lined vascular sinuses that resembled a cavernous hemangioma (Figure 2, A and B). The cells lining the papillary tufts were positive for the endothelial marker ERG (Figure 2C), which supported the morphologic diagnosis of papillary endothelial hyperplasia. These changes are nonspecific, and similar lesions are observed in human blood vessels in association with thrombosis. The observation that the proliferating vascular elements surrounded the thrombus and still expressed SNF5 (Figure 2C) suggested the

hyperplastic endothelium likely represented a robust revascularization response to thrombosis and was not the cause of the hemorrhage. Although the papillary endothelial hyperplasia was likely reactive, it was possible the hemangioma-like proliferation reflected a benign neoplastic process.

Only one female *Vhl*^{2B/+} mouse developed an ovarian cyst (1/6 = 17%) (Table 1 and Figure 2, D–F). The mouse had an intact ovary with enlarged, cystic, and hemorrhagic follicles and large, dilated, hemorrhagic cysts (Figure 2, D and E). The cells lining the hemorrhagic cysts maintained SNF5 expression (Figure 2F). In the past, examining >50 aged (>12 months) *Vhl*^{2B/+} female mice did not reveal any ovarian abnormality.¹¹ However, in the setting of *Snf5* deficiency, a subtle vascular abnormality, possibly induced by modestly activated HIF1 signaling, may have resulted in ovarian hemorrhage, dissection into, and filling of normal cystic ovarian structures (eg, tertiary ovarian follicles), with subsequent thrombus formation and obliteration of the ovarian parenchyma.

Compared with normal mouse ovaries (Figure 3, A and B), affected ovaries in the *Snf5*^{+/-};*Vhl*^{2B/+} mice (Figure 3,

Figure 3 Histopathology of normal mouse ovary (OV) and ovarian cysts from *Snf5*^{+/-};*Vhl*^{2B/+} mice. **A:** Normal ovaries from a C57BL/6 mouse showing follicles (F) at various stages of development, separated by ovarian stromal cells. **B:** Represents the area marked by the black box in **A**. **C:** Representative early thrombus (T) from a *Snf5*^{+/-};*Vhl*^{2B/+} mouse. **D:** The area marked by the black box in **C**. **E:** Representative organized thrombus from a *Snf5*^{+/-};*Vhl*^{2B/+} mouse. **F:** The area marked by the black box in **E**. Note the progression of distortion and obliteration of normal ovarian parenchyma by hemorrhagic pseudocysts and thrombi in the early and organized samples. Original magnification: $\times 40$ (**A**, **C**, and **E**); $\times 200$ (**B** and **D**); $\times 400$ (**F**). B, ovarian bursa; GC, granulosa cells (yellow circle); H, hemorrhage; Ovi, oviduct; OSE, ovarian surface epithelium.

C–F) contained large cystic spaces with hemorrhage and thrombus formation, surrounded by residual ovarian follicles, within the confines of the ovarian surface epithelium and ovarian bursa. In other mice, the hemorrhage extended beyond the ovary and involved adjacent adipose tissue (data not shown). A range of pathologies were observed among the ovaries in these mice, including early (Figure 3, C and D) and organized (Figure 3, E and F) thrombi. The hemorrhagic areas were focally lined by cuboidal cells that resembled granulosa cells and lacked a surrounding smooth muscle layer to suggest they represented dilated blood vessels. The absence of a definite epithelial or endothelial lining in the hemorrhagic areas in *Snf5*^{+/-};*Vhl*^{2B/+} mice indicated that, histologically, they represented hemorrhagic pseudocysts and not true cysts. Only one *Snf5*^{+/-};*Vhl*^{2B/+} mouse displayed a lesion consistent with a hemangioma, a common disease manifestation in patients carrying one copy of mutated *VHL*. The vascular pathology underlying the hemorrhage was not clear.

A range of SNF5 expression was observed in ovarian parenchymal cells in *Snf5*^{+/-};*Vhl*^{2B/+} mice, ranging from strong expression to almost complete absence (data not shown). This mosaic pattern of SNF5 expression was generally not observed in MRTs that arose in *Snf5*^{+/-} mice^{20–22} (Figure 1). The presence of SNF5-negative cells

in wild-type (Figure 2, G and H) and *Vhl*^{2B/+} (Figure 2F) mouse ovaries supported the hypothesis that they represented ovarian cells that normally lack SNF5 or express levels below the threshold for detection by IHC. However, for *Snf5*^{+/-} mice the possibility that they may represent proliferation of cells that lost the second *Snf5* allele cannot be ruled out. These mechanisms are not mutually exclusive. Regardless, the increased prevalence of ovarian cysts in the *Snf5*^{+/-};*Vhl*^{2B/+} mice may reflect changes in the tissue microenvironment associated with the SNF5-negative stromal cells.

Discussion

Hemorrhagic cystic ovaries have been described in several other genetic backgrounds. Targeted inactivation of the BRG1/SMARCA4 ATPase subunit of the SWI/SNF complex, with or without the mutually exclusive ATPase subunit BRM/SMARCA2, leads to cystic and hemorrhagic ovarian follicle formation in >60% of the mice.¹⁷ In addition, mice deficient for steroidogenic factor 1 (Sf1/Nr5a1),²⁴ aromatase,²⁵ and estrogen receptor (ER) α and ER β , either alone or in combination,²⁶ all develop hemorrhagic ovarian follicles. Cyp19 is an Sf1 target gene in mouse testis and

likely in the ovary where it is specifically expressed in ovarian granulosa cells^{25,27} and converts androgens produced by adjacent theca cells into estrogens. In addition to the endocrine effects, estrogens regulate transcription in granulosa cells by ER α and ER β . ER α is known to interact with the SWI/SNF complex by BRG1.^{28–30} Therefore, defects in core members of the SWI/SNF complex—either SNF5 or BRG1/SMARCA4—may impair transactivation of estrogen-regulated genes in granulosa cells, resulting in hemorrhagic ovarian follicles. The mechanisms involved in this pathology and the contribution of altered VHL signaling remain unclear.

Our results contribute to the growing association between mutation of genes encoding SWI/SNF complex subunits and ovarian cysts and carcinomas. Recent reports have established *SMARCA4* mutations as the driving event in a rare type of ovarian cancer, small cell carcinoma of the ovary, hypercalcemic type,^{13,15,16,19} and in dedifferentiated ovarian and endometrial carcinomas.^{31–34} Of interest, a small subset of MRTs displayed *SMARCA4* inactivation rather than *SNF5*^{35,36}; similarly, the dedifferentiated endometrial and ovarian carcinomas that have wild-type *SMARCA4* instead have mutations in *ARID1A* and *ARID1B*.³⁷

Why do mutations in members of the SWI/SNF complex, including SNF5, ARID1A, and SMARCA4, lead to cysts and tumors of the gynecologic tract? One possibility is that the chromatin alterations induced by aberrant SWI/SNF complex activity cooperate with enhanced local angiogenesis contributed by the heterozygous *Vhl* mutation to promote a novel change in gene expression.⁴ Interestingly, a previous report found that mice with low expression of the BAF155/SMARCC1 subunit display defects in angiogenesis during prenatal development.³⁸ Furthermore, a recent report found that the SMARCA4 and SMARCA2 ATPases may serve as coactivators for a subset of HIF genes.⁹ Therefore, the modest activation of HIF signaling that occurs with the *Vhl* mutation would activate a different repertoire of genes on changes in SWI/SNF complex activity that favor diseases within the ovary. Alternatively, permissive angiogenesis that results from low constitutive HIF1 expression, or another unknown feature of heterozygous *Vhl* mutation, may create a favorable environment for cyst development after SNF5 loss in the ovary. Ovarian defects have not been reported to date in VHL disease kindreds, and *VHL* loss or mutation has not been reported in conjunction with ovarian cystic disease. Recent studies, however, have linked *VHL* mutation with ovarian cancer aggressiveness, by HIF1- α activation, suggesting that the impact of downstream dysregulated hypoxia signaling can play a role in ovarian tissues.³⁹

Taken together, these findings suggest that *Snf5* mutation—associated phenotypes can be influenced by variants in other cancer-associated genes. In this case, a heterozygous mutation in *Vhl*, a hypomorph typically associated with a mild HIF stabilization effect, and virtually no tumor phenotype alone, cooperated with *Snf5* deletion to

produce hemorrhagic ovarian cysts. These data further support the role of mutations in SWI/SNF complex components in a broad range of human diseases, as well as the potential for host variant genetic features to alter these disease phenotypes.

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