Disruption of tubular Flcn expression as a mouse model for renal tumor induction

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The study of kidney cancer pathogenesis and its treatment has been limited by the scarcity of genetically defined animal models. The *FLCN* gene that codes for the protein folliculin, mutated in Birt–Hogg–Dubé syndrome, presents a new target for mouse modeling of kidney cancer. Here we developed a kidney-specific knockout model by disrupting the mouse *Flcn* in the proximal tubules, thus avoiding homozygous embryonic lethality or neonatal mortality, and eliminating the requirement of loss of heterozygosity for tumorigenesis. This knockout develops renal cysts and early onset (6 months) of multiple histological subtypes of renal neoplasms featuring high tumor penetrance. Although the majority of the tumors were chromophobe renal cell carcinomas in affected mice under 1 year of age, papillary renal cell carcinomas predominated in the kidneys of older knockout mice. This renal neoplasia from cystic hyperplasia at 4 months to high-grade renal tumors by 16 months represented the progression of tumorigenesis. The mTOR and TGF-β signalings were upregulated in Flcn-deficient tumors, and these two activated pathways may synergetically cause renal tumorigenesis. Treatment of knockout mice with the mTOR inhibitor rapamycin for 10 months led to the suppression of tumor growth. Thus, our model recapitulates human Birt–Hogg–Dubé kidney tumorigenesis, provides a valuable tool for further study of *Flcn*-deficient renal tumorigenesis, and tests new drugs/approaches to their treatment.

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Genetically engineered mouse models have been proven to be useful in validating gene functions, gaining insight into the pathogenesis of diseases, and providing better clinical models in which to test novel therapeutic strategies. However, studies of renal cell carcinoma (RCC) have been limited by the scarcity of genetically defined animal models. RCC is a heterogeneous disease consisting of histopathologic subtypes including clear cell RCC (CCRCC), papillary RCC (PRCC), chromophobe RCC (CRCC), collecting duct carcinoma, and unclassified RCC. There are several hereditary conditions that predispose to RCC: von Hippel–Lindau (VHL)[1] syndrome, hereditary papillary renal carcinoma type 1 (ref. 2), hereditary leiomyomatosis and renal cell cancer,[3,4] tuberous sclerosis, hereditary hyperparathyroidism-jaw tumor,[5] and Birt–Hogg–Dubé (BHD) disease.[6] Although VHL is specifically susceptible to CCRCC, hereditary papillary renal carcinoma type 1, and hereditary leiomyomatosis and renal cell cancer tend to develop PRCC type 1 and PRCC type 2, respectively. In addition, clear cell PRCC was identified in tuberous sclerosis. Hereditary hyperparathyroidism-jaw tumor is associated with

Wilms tumor and BHD inclines to produce all types of RCCs. Studies into these conditions led to the identification of crucial RCC-related genes such as *VHL* (VHL), *c-Met* (hereditary papillary renal carcinoma type 1), *FH* (hereditary leiomyomatosis and renal cell cancer), *TSC1/TSC2* (tuberous sclerosis), *HRPT2* (hereditary hyperparathyroidism-jaw tumor), and *FLCN* (BHD).[1,2,4,7–9] Yet once these RCC-related genes were recognized, efforts to recapitulate human RCC by disrupting these genes in the mouse were less than successful. The *VHL* gene, identified inVHL syndrome, is mutated in ~70% of CCRCC cases, but disruption of the *Vhl* in mice failed to produce key manifestations of the human disease. Attempts to recapitulate human kidney carcinogenesis by conditional knockout (KO) of the *Vhl* in the mouse kidney were not successful, although kidney cysts/hyperplasia were observed in affected mouse kidneys.[10–13] KOs of *c-Met* and *Fh* in mouse kidney do not develop kidney tumors. Other mouse models generated through genetic disruption of the kidney cancer–related genes *Tsc1/Tsc2*, *Apc*, and *Pten* either presented difficulties in studying renal tumorigenesis because of a high neonatal mortality (*Apc*) or a long latency in tumor development (*Tsc1*), or lack of tissue-specific targeting (*Tsc-1* and *Tsc-2*).[14–19] The inability of mouse models to recapitulate renal tumors has long puzzled kidney cancer researchers. Clearly, the development of an animal model that recapitulates human RCC is needed to advance basic and preclinical studies of renal epithelial neoplasia.

The identification of the *FLCN* gene—[8]which codes for the protein folliculin (FLCN) and is associated with Birt–Hogg–Dubé (BHD) disease—[6]brought new hope to RCC mouse modeling. BHD is characterized by lesions in multiple organs, including skin tumors, RCC, colon polyps/cancer, spontaneous pneumothorax, and lung cysts.[6] Although VHL, hereditary papillary renal carcinoma type 1, and hereditary leiomyomatosis and renal cell cancer are each associated with only one specific histological subtype of kidney cancer, patients with BHD develop multiple histological types of renal cell neoplasm including CCRCC, PRCC, CRCC, oncocytoma, and chromophobe/oncocytoma hybrid,[20] implying that the *FLCN* gene has a more universal suppressive role in the formation of kidney cancer.[20] As RCC is one of the main features of BHD and because patients with BHD develop multiple subtypes of RCC,[20,21] *FLCN* could be a potent target in a genetically engineered kidney cancer mouse model that covers the entire spectrum of renal tumors. Moreover, causative alterations of the orthologous *Flcn* were detected in other mammalian RCCs and renal cysts (e.g., dogs and rats),[22,23] which suggested that development of a *Flcn* KO mouse model was feasible.

Three groups have previously developed *Flcn* conventional KO mouse models.[24–26] Similar to the natural rat and dog models,[22,23] these mouse models present homozygous embryonic death and the heterozygotes developed kidney cysts and solid tumors. For instance, Hasumi *et al.*[24] showed that their heterozygous mice developed a wide spectrum of kidney tumors including clear cell, papillary, hybrid of CRCC and oncocytic cells, and oncocytoma, mimicking the kidney tumor phenotype found in humans with BHD. However, those heterozygotes depend on environmental conditions to generate the second genetic alteration (e.g.,), which leads to late-onset of tumorigenesis (e.g., median tumor-free survival: 25 months, close to the domestic mouse life span),[24] uncertain penetrance, or lack of tissue specificity. Thus, those models are not suitable for drug test/development for BHD. To avoid the embryonic death of homozygotes, we and another group generated a kidney-specific KO mouse model (*Flcn*$^{flox/flox}$/*Ksp-Cre*) through disruption of the *Flcn* gene in the distal tubules, collecting ducts, and the thick ascending limb of Henle's loop.[27,28] Unfortunately, the affected mice rapidly developed polycystic kidney disease after birth and died within 3 weeks, probably owing to disruption of *Flcn* in multiple types of kidney tubules.

To obtain a kidney tissue–specific KO mouse model having an extended life span and being suitable for preclinical applications, we have generated a new mouse model by disrupting the *Flcn* gene in only the kidney proximal tubules. The affected mice had a much longer lifespan than *Flcn*$^{flox/flox}$/*Ksp-Cre* mice[27,28] and developed a wide spectrum of kidney tumors earlier, representing the first kidney proximal tubule KO that recapitulates the kidney tumorigenesis of the human BHD.

RESULTS

Kidney proximal tubule–specific *Flcn* KO mice develop multiple renal cysts

To generate kidney proximal tubule–specific *Flcn*-deficient mice, *Flcn*$^{flox/flox}$ mice were bred to *Sglt2-Cre* transgenic mice, with expression of Cre recombinase under the control of the kidney proximal tubule–specific *Sglt2* gene promoter.[29] The resulting mice were kidney proximal tubule–directed *Flcn* homozygous KOs *Flcn*$^{flox/flox}$/*Sglt2-Cre*. These mice developed bilateral cystic kidneys, with more than half of them starting to develop cysts at 1 month (Figure 1a); their cysts appeared

Table 1 | Phenotype of three genotypic groups of mice

Strains	KO ($n=100$[a])				Het ($n=33$)				WT ($n=38$)			
Age (months)	1–6	7–12	13–24	>24	1–6	7–12	13–24	>24	1–6	7–12	13–24	>24
Mice with lesions	10	43	41	0	0	0	0	5	0	0	0	1
Mice with tumors	3	23	27	0	0	0	0	2	0	0	0	0
Total number of mice	16	43	41	0	4	5	6	18	6	9	11	12

Het, *Flcn*$^{flox/+}$/*Sglt2-Cre*; KO, *Flcn*$^{flox/flox}$/*Sglt2-Cre*; WT, *Flcn*$^{flox/flox}$.
For those mice, cysts or tumors were identified under dissection microscopy, and small tumors might be missed without histological analysis.
[a]Twenty-four mice were found dead with light decomposition.