Tumor suppressor FLCN inhibits tumorigenesis of a FLCN-null renal cancer cell line and regulates expression of key molecules in TGF-β signaling

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Abstract

Background: Germline mutations in the FLCN gene are responsible for the development of fibrofolliculomas, lung cysts and renal neoplasia in Birt-Hogg-Dubé (BHD) syndrome. The encoded protein folliculin (FLCN) is conserved across species but contains no classic motifs or domains and its function remains unknown. Somatic mutations or loss of heterozygosity in the remaining wild type copy of the FLCN gene have been found in renal tumors from BHD patients suggesting that FLCN is a classic tumor suppressor gene.

Results: To examine the tumor suppressor function of FLCN, wild-type or mutant FLCN (H255R) was stably expressed in a FLCN-null renal tumor cell line, UOK257, derived from a BHD patient. When these cells were injected into nude mice, tumor development was inversely dependent upon the level of wild-type FLCN expression. We identified genes that were differentially expressed in the cell lines with or without wild-type FLCN, many of which are involved in TGF-β signaling, including TGF-β2 (TGFB2), inhibin β A chain (INHBA), thrombospondin 1 (THBS1), gremlin (GREM1), and SMAD3. In support of the in vitro data, TGFB2, INHBA, THBS1 and SMAD3 expression levels were significantly lower in BHD-associated renal tumors compared with normal kidney tissue. Although receptor mediated SMAD phosphorylation was not affected, basal and maximal TGF-β-induced levels of TGFB2, INHBA and SMAD7 were dramatically reduced in FLCN-null cells compared with FLCN-restored cells. Secreted TGF-β2 and activin A (homo-dimer of INHBA) protein levels were also lower in FLCN-null cells compared with FLCN-restored cells. Consistent with a growth suppressive function, activin A (but not TGF-β2) completely suppressed anchorage-independent growth of FLCN-null UOK257 cells.

Conclusions: Our data demonstrate a role for FLCN in the regulation of key molecules in TGF-β signaling and confirm deregulation of their expression in BHD-associated renal tumors. Thus, deregulation of genes involved in TGF-β signaling by FLCN inactivation is likely to be an important step for tumorigenesis in BHD syndrome.

Background

Birt-Hogg-Dubé (BHD) syndrome is a familial disorder that predisposes patients to develop hair follicle hamartomas (84-90% penetrance), lung cysts (85% penetrance) and renal neoplasia (29-34% penetrance) [1-5]. BHD patients are at risk to develop bilateral, multifocal renal tumors with a variety of histologies, mainly chromophobe (34%) and oncocytic hybrid (50%) tumors with features of both chromophobe renal cell carcinoma (RCC) and renal oncocytoma. Clear cell and papillary RCC as well as renal oncocytomas are also found in BHD patients at a low frequency [6]. The BHD syndrome locus was mapped to chromosome 17p11.2 by linkage analysis in BHD families, and germline mutations in a novel gene FLCN (alias BHD), were identified and characterized [5,7-11]. Most BHD families carry germline mutations predicted to truncate the encoded protein, folliculin (FLCN), including insertion/deletion, nonsense, and splice-site mutations reported in several large BHD cohorts [4,5,11]. Either somatic "second hit" mutations predicted to truncate the protein or loss of heterozygosity at the BHD syndrome locus was identified in 70% of renal tumors from...
BHD patients [12] supporting a tumor suppressor function for FLCN.

Two naturally-occurring animal models have been described that show phenotypes similar to BHD patients. The Nihon rat model develops renal carcinoma with clear cell histology by 6 months of age and harbors a cytosome insertion mutation in exon 3 of rat Flcn [13]. A canine model of BHD, which develops renal cystadenocarcinoma and nodular dermatofibrosis (RCND), carries a germline missense mutation (H255R) in canine Flcn [14]. Recently, we and others described a conditional Flcn knockout mouse model in which Flcn inactivation was targeted to mouse kidney using the Cre-lox site-specific recombination system. The affected mice displayed renal hyperplasia, formation of multiple cysts and renal dysplasia, and renal tumors derived from Flcn-null mice [15,16]. In addition, the renal tumors from mice described by Hartman et al. showed reduced phosphorylation of both mTORC1 and mTORC2 pathways in kidney tumors from Flcn<sup>−/−</sup> mice [17]. Hudon et al. suggest that up or down regulation of mTOR by inactivation of Flcn in a mouse model may be context-dependent [19]. Thus it is possible that mTOR signaling is regulated differently by FLCN depending on cell types or experimental conditions.

A renal cancer cell line (UOK257) established from a BHD patient was recently developed and characterized [23]. UOK257 cells harbor a cytosome insertion in a (poly)C tract, the frequently mutated "hot spot" within exon 11 of FLCN (c.1285dupC), and have lost the wild-type copy of FLCN. Cytogenetic analysis revealed that the cell line was nearly triploid displaying multiple unbalanced translocations and deletions of chromosomes. The MYC copy number was heterogeneous in UOK257 cells ranging from 3 to 5 copies. These cells formed tumors in immunodeficient mice (SCID/BEIG) exhibiting predominantly atypical clear epithelial cell type histology, as well as a variety of other histologic types including tubular papillary, and foci reminiscent of chromophobe RCC, all of which resemble the histologies within the tumor from which the cell line was derived [23].

In the current study, in order to investigate the tumor suppressor function of FLCN we have introduced wild-type FLCN into UOK257 cells and compared their growth in vitro and in vivo. We found that wild-type FLCN suppressed tumor cell growth in vitro, confirming the tumor suppressor function of FLCN. In addition, we employed gene expression microarray analysis to identify novel downstream target genes of FLCN. Among the differentially expressed genes, we identified several critical genes involved in TGF-β signaling including TGFBR2, INHBA, THBS1, GREM1 and SMAD3. Since deregulation of TGF-β signaling is important in tumorigenesis and tumor progression, we characterized the expression of these genes in FLCN-null and FLCN-expressing cultured cells as well as in renal tumors surgically removed from BHD patients. In addition, we examined the growth suppressive effect of activin A in the FLCN-null cell line and investigated receptor mediated TGF-β signaling in FLCN-null and FLCN-restored cell lines.

**Results**

**Wild-type FLCN expression was restored in UOK257 cells by lentiviral vectors.**

To evaluate the tumor suppressor function of FLCN, wild-type or mutant (H255R) FLCN cDNA was introduced into the FLCN-null UOK257 cells using lentiviral vectors. Four clones expressing wild-type FLCN (UOK257-2, -3, -4, and -6) and one clone expressing mutant FLCN-H255R (UOK257-H255R; Flcn missense mutation responsible for canine RCND) were isolated and compared to the parental UOK257 cells (UOK257-P). FLCN protein expression was measured by Western blot analysis using a mouse monoclonal anti-FLCN antibody. Relatively high levels of FLCN protein were detected in the UOK257-2, -4 and -6 cells but very low levels of FLCN protein were detected in the UOK257-3 and UOK257-H255R cells (Fig. 1A).

**FLCN mRNA expression**
from both the transgene and endogenous FLCN was measured by quantitative RT-PCR. The total FLCN mRNA expression was increased by the expression of the wild-type FLCN or mutant FLCN-H255R transgene to varying degrees in the cell lines (P = 3 < 4 < H255R <2 <6) (Fig. 1B).

Anchorage independent but not dependent growth of UOK257 cells was inversely correlated with wild-type FLCN expression

We examined whether introduction of wild-type or mutant FLCN could affect anchorage dependent and independent growth of UOK257 cells. Anchorage dependent growth of UOK257 cells on culture dishes was not affected by the expression of wild-type or mutant FLCN (Fig. 1C). However, anchorage independent growth measured as colony numbers on soft agar was low in the wild-type FLCN cell line UOK257-2, which expressed high levels of FLCN, compared to the parental UOK257 cell line (UOK257-P) (Fig. 1D). One of the characteristics of the UOK257 cells was a slow growth rate (doubling time, 52+/- 9 hrs) on culture dishes. These cells also grew very slowly in soft agar taking 3-4 weeks to reach a countable colony size. By comparison, HT-1080 cells derived from a fibrosarcoma grew faster in soft agar and often generated larger colonies (data not shown).

Tumor growth was suppressed by wild-type FLCN but not by mutant FLCN-H255R expression

To examine whether the tumorigenic potential of UOK257 cells was affected by wild-type or mutant FLCN, mutant FLCN (UOK257-P and -H255R) and wild-type FLCN-expressing cells (UOK257-2, -3, -4 and -6) were injected subcutaneously with matrigel into athymic nude mice and tumor growth was measured for up to one year. Most of the mice injected with UOK257-P and UOK257-H255R cells developed solid tumors, although some animals only developed flat patches of tumor cells (See addi-
tional file 1: Table S1 and Fig. 2A). All of these tumors were high grade and exhibited clear cell histology (See additional file 1: Table S1 and Fig. 2B). On the other hand, the mice injected with UOK257 cells expressing a high level of FLCN (UOK257-2, -4 and -6) did not develop tumors (See additional file 1: Table S1 and Fig. 2A). Instead, flat masses of cells only rarely containing tumor cells were observed in 6 of 35 (17%) of the animals. The mice injected with UOK257-3 cells expressing a very low level of FLCN developed solid tumors with low incidence (2/10) and smaller size (See additional file 1: Table S1). In some animals, UOK257-3 cells grew as flat patches (5/10; Fig. 2A) and exhibited mostly clear cell histology with varying grades (low to high; Fig. 2B).

We investigated whether wild-type or mutant FLCN transgenes, or the endogenous mutant FLCN genes were lost during tumor progression. Genomic DNA was isolated from the tumors or tumor cell patches and PCR was performed using a primer pair specific to exon 10 and exon 11 that amplifies 664 bp of the endogenous FLCN gene or 99 bp of the FLCN transgene. All of the tumors from the cell lines retained the endogenous mutant FLCN gene (c.1285dupC) and all of the tumors from UOK257-3 and UOK257-H255R retained their respective transgenes (See additional file 1: Fig. S1).

Gene expression microarray analysis identified genes regulated by FLCN and the pathways in which they interact
To identify the genes regulated by FLCN expression, we performed gene expression microarray analysis using RNAs isolated from the UOK257 cell lines expressing either no, mutant or wild-type FLCN. We identified a total of 439 genes, which were up or down-regulated more than 2-fold in the mutant and FLCN-null cell lines (UOK257-P and UOK257-H255R) compared to the wild-type FLCN cell lines (UOK257-2, -3, -4 and -6) (See additional file 2: Table S2). To explore the biological processes and pathways regulated by FLCN, the genes were subclassified with the help of "Panther Classification System" http://www.pantherdb.org and three prominent pathways were identified, namely cadherin signaling, TGF-β signaling, and angiogenesis (Fig. 3A). Although all three of these pathways are important in tumorigenesis, we focused on the genes involved in TGF-β signaling (See additional file 1: Fig. S2). We found that TGF-β2 (TGFB2), Inhibin β A (INHBA, a subunit of activin A), SMAD3 (SMAD3) and thrombospondin-1 (THBS1) were down-regulated, and Gremlin (GREM1) was upregulated in FLCN-null and mutant FLCN-H255R UOK257 cells compared with FLCN-restored UOK257 cells. We confirmed the GREM1, TGFB2, INHBA, SMAD3 and THBS1
microarray results by quantitative RT-PCR (See additional file 1: Fig. S3).

**Knockdown of FLCN deregulates TGFB2, INHBA, GREM1, THBS1 and SMAD3 expression in FLCN-restored UOK257 cells**

We next examined whether the expression levels of TGFB2, INHBA, THBS1, GREM1 and SMAD3 could be deregulated by knockdown of FLCN in FLCN-restored UOK257 cells. A FLCN-knockdown cell line was generated by introducing a retrovirus that expressed shRNA against FLCN in FLCN-restored UOK257 cells. A FLCN-knockdown cell line was generated by introducing a retrovirus that expressed shRNA against FLCN in FLCN-restored UOK257 cells. In addition to reduced expression of FLCN, the expression of TGFB2, INHBA, THBS1 and SMAD3 was decreased and the expression of GREM1 was increased in the FLCN-knockdown cell line (Fig. 3B).

**GREM1, TGFB2, INHBA, THBS1 and SMAD3 expression levels were down-regulated in BHD-associated renal tumors**

In order to determine whether the genes that were regulated by FLCN in *in vitro* cell culture were differentially expressed in renal tumors from BHD patients compared to normal kidney parenchyma, we performed quantitative RT-PCR using RNA isolated from these tissues. GREM1, TGFB2, INHBA, THBS1 and SMAD3 RNA expression levels were significantly lower in the BHD renal tumors compared to normal kidney tissue (Fig. 4A). However, FLCN RNA levels were not statistically different (*P* = 0.316). In support of the RT-PCR data, immunohistochemical staining of TGFB-β2 showed strong TGFB-β2 expression in the normal renal tubules but reduced expression in the tumors from BHD patients (Fig. 4B, left panel). In addition, the UOK257 xenograft tumors expressed lower levels of TGFB2 compared to normal mouse kidney (Fig. 4B, right panel).

We measured protein expression of SMAD2, SMAD3, phospho-SMAD3 (pSMAD3) and FLCN in renal tumors from BHD patients (*n* = 11) and normal human kidney tissue (*n* = 5). pSMAD3 levels were high in 3 out of 5 normal kidneys but only 1 (T11) of 11 tumors (Fig. 4C). In addition, SMAD3 levels and SMAD3/SMAD2 ratios were higher in normal kidneys compared to the tumors. On the other hand FLCN protein levels were lower or undetectable in all tumors except T11, in which a moderate level of FLCN expression was detected along with high levels of pSMAD3 and SMAD3 (Fig. 4C). Therefore it is likely that the T11 tumor was contaminated with normal kidney tissue.

In order to investigate whether receptor mediated TGFB-β signaling was disrupted by the loss of FLCN expression, TGFB-β induced SMAD3 phosphorylation was
Figure 4 Genes involved in TGF-β signaling and the encoded proteins dysregulated in renal tumors from BHD patients. (A) Quantitative RT-PCR of TGFB2, INHBA, SMAD3, THBS1, GREM1 and FLCN expression levels in human BHD tumors (T, n = 12) and normal kidneys (N, n = 8). Gene expressions were normalized against cyclophilin gene expression and relative expressions were calculated against gene expression in UOK257 cells. Median values of expression levels were indicated with short lines. Points, mean expression of each sample. P value, Mann-Whitney U-test. (B) Reduced expression of TGF-β2 in the BHD tumors (left panel) and the UOK257 xenograft tumors (right panel) compared to normal renal tubules as shown by immunohistochemical staining. Five BHD tumors were examined and a representative immunostained image is shown. (C) Positive correlation of pSMAD3 and SMAD3 with FLCN expression in human BHD kidney tumors and normal kidney tissue. pSMAD3, SMAD2, SMAD3 and FLCN protein levels were measured by western blot analysis in normal kidney tissue (n = 5) and BHD tumors (n = 11).
examined in UOK257 cells and compared to UOK257-2 cells. TGF-β induced SMAD3 phosphorylation was not affected by FLCN inactivation (Fig. 5A). In addition, BMP4 induced SMAD1/5/8 phosphorylation was not disrupted by loss of FLCN expression (data not shown). We then examined whether TGF-β induced gene expression was dysregulated in FLCN-null UOK257 cells. SMAD7, an inhibitory SMAD, is known to be induced by TGF-β. SMAD7 expression was induced in both UOK257 and UOK257-2 cell lines (Fig. 5B). However the basal and the maximal induced levels of SMAD7 were two fold greater in UOK257-2 cells than in UOK257 cells. Similar to SMAD7, TGFB2 and INHBA expressions were induced by TGF-β in both cell lines but their basal and maximal levels of expression were substantially higher (5.8- to 23-fold) in FLCN-null UOK257-2 cells compared with UOK257-2/FLCN-KD cells (Fig. 5C(a) and Fig. 5C(b)). Similar results were obtained from an experiment using UOK257-2/FLCN-KD cells. TGF-β2 or activin A and cultured for 4 weeks in soft-agar. Unexpectedly, TGF-β2 appeared to increase colony formation of UOK257 cells at both 1 ng/ml and 5 ng/ml (Fig. 6C). However, activin A reduced colony formation at 1 ng/ml and completely suppressed colony formation at 5 ng/ml (Fig. 6D).

Discussion

UOK257 is the only renal cancer cell line available to date that has been established from a BHD patient’s tumor tissue. This cell line is particularly valuable for study of the biological role of FLCN inactivation in tumorigenesis because it harbors a FLCN mutation predicted to produce only truncated mutant protein and induces the growth of tumors in vivo with histology resembling the BHD-associated renal tumor from which it was derived [23]. In this study, we have established and characterized UOK257 cell lines in which wild-type or mutant FLCN was stably expressed. Although anchorage dependent cell growth in vitro was not affected by wild-type FLCN expression, cell growth in vivo and anchorage-independent growth in soft agar were severely diminished by the expression of wild-type FLCN. We have searched for downstream target genes regulated by FLCN through gene expression microarray analysis and identified a number of genes that were differentially expressed in wild-type FLCN (UOK257-2, -4, and -6) compared with mutant FLCN and FLCN-null (UOK257-H255R and -P) cells. We found three prominent groups of genes involved in cadherin signaling, TGF-β signaling, and angiogenesis. Notably, several key genes involved in TGF-β signaling, such as TGFB2, INHBA, THBS1 and SMAD3, were down-regulated in FLCN-null and mutant FLCN cells as well as in the BHD-associated renal tumors. Consistently, GREM1, the antagonist of BMP that signals through SMADs was highly up-regulated in mutant FLCN and FLCN-null UOK257 cells although its expression was low in BHD-associated renal tumors.

We observed that the expression level of FLCN is important for tumor suppression, since the UOK257 cell lines (UOK257-4, -2 and -6) expressing high levels of FLCN did not develop tumors whereas the UOK257-3 cell line expressing a very low level of FLCN, did develop tumors with a low incidence (2 out of 10). It is likely that the FLCN expression level in UOK257-3 cells is marginal for tumor suppression, allowing tumor growth in some animals but suppressing tumor growth in others. In support of this idea, the expression levels of the downstream target genes in UOK257-3 cells were either similar to FLCN-null and FLCN mutant cells (UOK257-P and UOK257-H255R), or midway between the FLCN-null-FLCN mutant group and the FLCN-restored group, which expressed high levels of FLCN (UOK257-2 and UOK257-6) (See additional file 1: Fig. S3).
Figure 5 TGF-β1 induced SMAD3 phosphorylation and effects of AICAR, Compound C and rapamycin on TGF-β1 induction of TGFβ2 and INHBA in FLCN-null and FLCN-expressing cells. (A) TGF-β1 induced SMAD3 phosphorylation in UOK257 and UOK257-2 cells. Cells were treated with 0, 0.1 and 1 ng/ml of TGF-β1 for 1 hr. (B) TGF-β1 induced TGFβ2, INHBA and SMAD7 mRNA expression in UOK257 and UOK257-2 cells. Cells were treated with 1 ng/ml of TGF-β1 for 0, 6, 12 and 24 hr after serum starvation for 12 hr. (C) (a and b) Cells were serum-starved for 24 hr and treated with 0.5 mM AICAR (AI), 20 μM Compound C (CC) or 1 nM rapamycin (Rapa) for 12 hr. UOK257-2/vector, UOK257-2 cells infected with empty retrovirus; UOK257-2/FLCN-KD, UOK257-2 cells infected with retrovirus expressing shRNA targeting FLCN. (c and d) Cells were serum-starved for 15 hr and treated with 0.5 mM AICAR or 20 μM Compound C (CC) for 6 hr.
Figure 6 Higher levels of secreted TGF-β2 and activin A proteins were detected in FLCN-restored UOK257-2 cells compared to the parental UOK257 cell line. (A) TGF-β2 and (B) activin A levels in cultured media were measured by ELISA (R&D systems). (C) TGF-β2 induced anchorage independent growth of UOK257 cells. However, (D) activin A suppressed anchorage independent growth of UOK257 cells. UOK257 cells (2,500 cells) were plated in soft-agar and cultured for 4 weeks in the presence of TGF-β2 or Activin A and stained with crystal violet. N = 4 for each treatment.
UOK257-H255R cells expressed a low level of FLCN protein resulting in loss of tumor suppressor function and deregulation of TGF-β signaling, even though they expressed slightly more FLCN mRNA than UOK257-4 cells (Fig.1A and 1B). These data suggest that FLCN-H255R missense mutant protein found in the canine model of BHD syndrome is less stable than wild-type FLCN. Thus decreased stability of mutant FLCN is likely to contribute to the loss of FLCN tumor suppressor function.

It has been suggested that Drosophila BHD (dBHD) regulates germline stem cell (GSC) maintenance downstream or in parallel with Jak/Stat and dpp (BMP ortholog in Drosophila) signaling [24]. dBHD knockdown by siRNA suppressed overproliferation of GSC induced by hyperactivation of Jak/Stat or dpp signaling. Interestingly, Jak1, encoding a kinase that transmits signals by phosphorylating Stats in cells, was identified by microarray analysis as a downregulated gene in the mutant FLCN and FLCN-null cells (Fig. 3A). We also identified several key genes in TGF-β/BMP signaling such as TGFβ2, INHBA, THBS1 and SMAD3 (a regulatory SMAD) that were down-regulated in the mutant FLCN and FLCN-null cells. On the other hand, GREM1, which encodes a protein that binds and inactivates BMP activity, was upregulated in the mutant and FLCN-null cells. Thus the genetic interactions between dBHD, and Jak/Stat and dpp (BMP) signaling may be partially explained by FLCN deregulation of genes involved in these pathways.

The human TGF-β superfamily consists of 42 members including TGF-βs, activins, bone morphogenetic proteins (BMPs), and growth and differentiating factors (GDFs) [25,26]. TGF-βs are multi-functional cytokines that modulate cell proliferation, apoptosis, differentiation, adhesion and migration. TGF-β shows a biphasic effect on tumor cell growth [27]. It inhibits tumor cell growth in the early phase of tumorigenesis but promotes cell growth when cells escape the anti-proliferative effect of TGF-β in the late phase of tumorigenesis. Interestingly, TGF-β2 induced anchorage independent growth of UOK257 cells (Fig. 6C), suggesting that UOK257 cells are refractory to the growth suppressive effect of TGF-β. The possibility exists that reduced expression of TGF-β2 in FLCN-null cells contributed to cell growth in the early phase of tumorigenesis.

Disruption of TGF-β signaling has been reported in many cancers. TGF-β type II receptor is often mutated in gastro-intestinal cancers [28-30]. Mutations in SMAD2 or SMAD4 occur frequently in pancreatic and colorectal carcinomas [31-33]. Although mutations in SMAD3 have not been reported, 3 out of 8 (37.5%) gastric tumors in one report showed low to undetectable levels of SMAD3 expression and restoration of SMAD3 suppressed tumorigenicity of gastric cancer cells [34]. Low levels of SMAD3 expression in the BHD tumors may contribute to the ability of these renal tumor cells to escape the growth suppressive effect of TGF-β.

Activins are homo- or heterodimeric proteins consisting of two β subunits (βA and βB), while inhibins are heterodimers of α and β subunits (inhibin-A [αβA] and inhibin-B [αβB]) [35]. INHBA is one of the β subunits (βA) that comprise activin A (βAβA), activin AB (βAβB) and inhibin A (αβA). Activin A regulates kidney organogenesis, tubular regeneration and renal fibrosis [reviewed in [36]]. Activins also induce apoptosis, and inhibit cell proliferation and tumor growth in numerous types of cells. In contrast to TGF-β2, activin A inhibited growth of UOK257 cells in soft-agar (Fig. 6D), suggesting that activin signaling is intact in UOK257 cells. Thus reduced expression of INHBA, β subunit of activin A, in UOK257 cells and BHD tumors, may be permissive for tumor cell growth. It would be interesting to examine whether activin A treatment can suppress BHD tumor growth in vivo.

Thrombospondin-1 (THBS1) is one of the five members of a family of thrombospondins that mediate the interaction of normal and cancer cells with the extracellular matrix and surrounding tissue. THBS1 suppresses tumor growth by activating TGF-β and by inhibiting angiogenesis. THBS1 exerts direct effects on endothelial cell migration and survival through interaction with CD36. It also reduces availability of VEGF by inhibiting MMP9, therefore releasing VEGF from the extracellular matrix. There are several reports suggesting that reduced expression of THBS1 or hypermethylation of THBS1 is associated with poor prognosis of cancer patients and higher tumor grade [37-40]. Accordingly THBS1 regulation may be an important part of the tumor suppressor function of FLCN.

We examined whether TGF-β signaling is dysregulated by the inactivation of the FLCN gene. TGF-β or BMP4 induced SMAD3 or SMAD1/5/8 phosphorylation was not affected by FLCN inactivation suggesting receptor mediated SMAD phosphorylation is not altered by FLCN. However, several genes whose expressions are regulated by TGF-β were dysregulated by the inactivation of FLCN. The basal and maximal induced levels of the downstream target genes (TGFβ2, INHBA and SMAD7) regulated by TGF-β were reduced in cells with FLCN inactivation. These data suggest that FLCN may regulate TGF-β signaling through a non-SMAD mediated mechanism. As a result of such regulation, the level of TGF-β ligands, such as TGF-β2 and activin A, could be highly induced in cells expressing FLCN by a positive feedback control.

A possible function of FLCN in energy sensing and metabolism has been suggested by its interaction with AMPK through FNIP1/2 and by the observation that
FLCN phosphorylation is affected by mTOR signaling (20-21). Here we demonstrated that an AMPK activator, AICAR, and an AMPK inhibitor, Compound C, as well as an mTOR inhibitor, rapamycin, affected the expression of the same key molecules involved in TGF-β signaling, which appear to be regulated by FLCN. Thus FLCN could be a key molecule connecting energy-sensing signals to growth suppressive TGF-β signaling.

**Conclusions**

Here for the first time we have confirmed the tumor suppressor function of *FLCN* in vivo and identified new potential FLCN downstream targets in the TGF-β signaling pathway. This study will provide a foundation for understanding the pathogenesis of BHD syndrome at the molecular level and be useful for finding therapeutic targets for treating BHD-associated kidney cancer and potentially, sporadic chromophobe RCC. We are currently analyzing the mechanism by which FLCN regulates these target genes and the functional importance of deregulation of these FLCN target genes in tumorigenesis.

**Methods**

**Establishment of cell lines, cell culture, and cell growth**

Wild-type or mutant (H255R) *FLCN* cDNA was transduced into UOK257 cells using the ViraPower Lentiviral expression system (Invitrogen) following the manufacturer’s protocols. Stable clones were selected using Blasticidin S (1.5 μg/ml). Cells were maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. To evaluate growth rate in culture, cells (2 × 10^5) were plated in each well of 96 well plates, cultured, and cell numbers were measured at day 1, 2, 3, 5 and 7 using the CyQuant Cell Proliferation Assay Kit (Molecular Probes). Adenoviral vectors (pAd/CMV/V5-DEST) expressing wild-type and mutant (c.1285dupC) *FLCN* were generated using the ViraPower Adenoviral Gateway system (Invitrogen) following the manufacturer’s protocol. A retroviral shRNA vector targeting *FLCN* was generated by inserting double stranded oligonucleotides (forward sequence, 5’-GATCCCC GGT-GTTTGAGGCAGAGCAGTGCTCTG CCTCAACACCTTTTATA-3’ and reverse sequence, 5’-GCTTAAAAAGGTTGGTTGAGGCGAGACGACTTCCTG TAAGCTTGCTTGCCTCAAACCCGGG-3’) into HindIII and BgIII sites of pSuper-Retro vector (Oligoengine) following the manufacturer’s instruction. UOK257-2 cells were infected with the *FLCN* shRNA vectors and selected against puromycin (7.5 μg/ml).

**Colonies formation assay**

UOK257 cells (5 × 10^3) were suspended in 1.5 ml of 0.3% agar in DMEM containing 10% FBS and were overlayed on 1.5 ml of pre-solidified 0.5% agar in the same medium. Cells were cultured in a CO₂ incubator for 3-4 weeks. Colonies were stained for 1 hour with 0.02% crystal violet solution dissolved in 10% neutral formalin. Colony number was counted under a dissection microscope after washing with PBS three times.

**Tumor growth in nude mice**

Cells (1 × 10^6) suspended with basement membrane matrix (BD Biosciences) were injected subcutaneously into the flanks of athymic nude mice. Tumor growth was measured once a week and mouse health was monitored daily. Mice bearing tumors larger than 2 cm, or showing severe health problems, were sacrificed and examined. Otherwise tumor growth was monitored for up to one year after injection. Tumors were fixed in 10% buffered formalin solution for histological examination and flash frozen in liquid nitrogen for protein and RNA extraction. Animal care procedures followed NCI-Frederick Animal Care and Use Committee guidelines.

**Immunoblotting**

Cells were harvested and lysed in RIPA buffer (50 mM Tris-Cl, pH 8.0 with 150 mM NaCl, 1.0% NP-40 and 0.5% sodium deoxycholate) or 1× SDS sample buffer (Biorad). Cell lysates were resolved by 4-20% SDSPAGE and blotted onto PVDF membrane. The following antibodies were used in this study: anti-FLCN mouse monoclonal [20], anti-β-actin (Sigma), anti-SMAD2/3 (Santa Cruz, sc-6032), and anti-pSMAD2/3 (Santa Cruz, sc-11769) antibodies. Immunoblots were processed by the ECL Detection System (Pierce) according to the manufacturer’s protocols.

**Immunohistochemistry**

Paraffin tissue sections were deparaffinized, rehydrated in graded alcohol and boiled in Tris-EDTA buffer pH 8.0 for 20 min at 90°C for antigen retrieval. After blocking, sections were probed with primary antibodies overnight and then incubated with HRP-polymer conjugated secondary antibodies. Diaminobenzidine hydrochloride (DAB) was used as a substrate for peroxidase. Sections were then briefly counterstained with hematoxylin and permanently mounted for observation.

**ELISA**

Cells (2 × 10^5) were cultured on 6 well plates for 3 days and culture media was collected for assay. TGF-β2, and activin A levels in the media were quantified by Human TGF-β2 DuoSet (R&D systems) and activin A DuoSet (R&D systems), respectively, following the manufacturer’s instruction.

**RNA isolation, microarray analysis and pathway analysis**

Total RNAs were isolated from the UOK257 cell lines using Trizol reagent (Invitrogen) and further purified using RNeasy mini kit (QIAGEN) according to the manu-
facturer’s protocols. Probes, which were generated using these RNAs, were hybridized to the Human Genome U133 Plus 2.0 arrays (Affymetrix) and processed according to recommended protocols. The CEL files were processed using the Partek Genomic Suite 6.2 (Partek Inc.). Data were transformed using a log normalization process and the differentially expressed genes were identified by Student's t-test and Mann-Whitney U-test. The genes that were differentially expressed in mutant FLCN cell lines (UOK257-P and -H255R) and wild-type FLCN cell lines (UOK257-2, -4 and -6) were used for further analysis.

Quantitative real-time reverse transcription-PCR (RT-PCR)

To confirm the microarray results, quantitative real-time reverse transcription PCR (RT-PCR) was performed. RNAs were digested with DNase I for 30 min at 37°C followed by heat denaturation at 70°C for 20 min to remove genomic DNA contamination. Total RNAs (2.5 μg) were primed with 100 ng random primers and reverse-transcribed by Superscript II reverse transcriptase (Invitrogen) at 42°C for 1 hr. The identical reactions were performed without reverse transcriptase to generate negative controls. PCR primers were generated using Primer 3 software [41] or Primer Express 3.0 (Applied Biosystems). Quantitative RT-PCR was performed with Power SYBR-Green or Taqman Gene Expression Master Mix (Applied Biosystems) using a 7300 Real-Time PCR system (Applied Biosystems) following the manufacturer's protocols. All reactions were run in triplicate using β-actin, GAPDH or cyclophilin A genes as internal controls. The relative level of a particular gene expression was evaluated according to the function of 2^-ddCt, where ddCt is dCt(treatment) - dCt(control), dCt is Ct(target gene) - Ct(GAPDH or actin) and Ct is the cycle at which the threshold is crossed. The gene-specific primer pairs for the PCR reactions are as follows: FLCN forward 5'-TTCCACGCCATTCCTA-CACCAGA-3' and reverse 5'-GCCCAACAGTGTGTACATT-CACTTG-3', GREM1 forward 5'-GCCAACACGCCAGCGCTTAA-3' and reverse 5'-TGATGTTGCGACTGTT-GCA-3', TGFβ2 forward 5'-CGAGAGGAGCGACGAA-CGC-3' and reverse 5'-AGGGGGCATGTCTA-TTTTG-3', THBS1 forward 5'-CCAGATCAGGCA-CACAGA-3' and reverse 5'-AGTTTCCGGTCTTATT-GAGG-3', INHBA forward 5'-TGGAGTGTGATGCGAAGGTCA-3' and reverse 5'-GCATGATGCCAGGGGACGCA-3', SMAD3 forward 5'-GACGGTCTGCTGGTGAATCC-3' and reverse 5'-GTGGCGTGGCACCACAAACCA-3', and GAPDH forward 5'-TTCCACCATG-GCAATTCC-3' and reverse 5'-CGCCCAATCTT-GATTGGGAGG-3', SMAD7 forward 5'-CACTGCTCAGA-3' and reverse 5'-CAGGCTCCAGAAGAAGTGG-3'. PCR product quality was monitored using post-PCR dissociation curve analysis.

Additional material

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

SBH designed the experiments. SBH, VAV, HBO, JS, DTN and MB performed the experimental work. MB generated critical cell lines for the work. MJM performed histopathologic analysis. SBH wrote the manuscript. WML and LSS contributed to the design of the experiments, review of the data, scientific discussions and manuscript editing. All authors read and approved the final manuscript.

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