

Folliculin regulates cyclin D1 expression through cis-acting elements in the 3' untranslated region of cyclin D1 mRNA

AKIKO KAWAI, TOSHIYUKI KOBAYASHI and OKIO HINO

¹Department of Pathology and Oncology, Juntendo University School of Medicine; ²Department of Molecular Pathogenesis, Juntendo University Graduate School of Medicine, Bunkyo-ku, Tokyo 113-8421, Japan

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Abstract. Birt-Hogg-Dubé syndrome (BHDS) is an autosomal dominantly inherited disease characterized by spontaneous pneumothorax, hair folliculomas and renal tumors. The responsible gene, *BHD*, is a tumor suppressor and encodes folliculin. Folliculin is an evolutionarily conserved protein (~67 kDa) with no apparent functional motif and its role has not yet been fully elucidated. In this study, we found that knockdown of *BHD* increased the levels of cyclin D1 in HeLa cells. A reporter assay with the cyclin D1 gene (*CCND1*) promoter region indicated that this increase was not caused by activation of transcription through known cis-acting elements. We examined the possibility of post-transcriptional mechanism using reporter constructs containing fragments of the cyclin D1 3' untranslated region (3'UTR). Transfection of control cells with a construct carrying a medial 1.3 kb 3'UTR fragment resulted in a significant reduction in luciferase activity. This effect was largely prevented by knockdown of *BHD*. Our results suggest that the post-transcriptional regulation of the *CCND1* expression by *BHD* may be associated with microRNA(s) or RNA binding protein(s) that bind to the 3'UTR.

Introduction

Birt-Hogg-Dubé syndrome (BHDS) is an autosomal dominantly inherited disease characterized by spontaneous pneumothorax, hair folliculomas and renal tumors (1). The mutation responsible for BHDS has been shown by positional cloning to occur in the *BHD* gene, which has tumor suppressor activity (2,3). We previously investigated hereditary renal carcinoma in the Nihon rat model and identified a germline mutation in *Bhd*, the rat homolog of *BHD* (4). Heterozygous Nihon mutant rats develop renal carcinomas that are mainly characterized by

clear-cell histology, while homozygotes for the mutation are embryonic lethal (5).

Human *BHD* is located on chromosome 17p11.2 and encodes folliculin, an evolutionarily conserved protein (~67 kDa) that has no apparent functional motif (2). Although the function of folliculin has not been fully elucidated, two folliculin-interacting proteins, folliculin-interacting protein 1 (FNIP1) and its homolog folliculin-interacting protein 2 (FNIP2/FNIPL), have been identified (6-8). Baba *et al* demonstrated that 5' AMP-activated protein kinase (AMPK) interacts with FNIP1 and phosphorylates both FNIP1 and folliculin (6). They also reported the phosphorylation of folliculin is regulated directly or indirectly by mTOR. These findings suggest that folliculin is involved in energy and nutrient sensing through the AMPK and mTOR signaling pathways.

In the present study, we found that an increase in cyclin D1 protein levels was caused in HeLa cells by knockdown of *BHD*. Recently, it was reported that cyclin D1 protein levels were elevated in renal cell tumors of *Bhd* heterozygous knockout mice and renal cysts of kidney-specific *Bhd* knockout mice, and that the absence of folliculin resulted in an increase in *CCND1* (cyclin D1 gene) mRNA levels in a *BHD*-deficient renal tumor cell line (UOK257) (9-11). *CCND1* is an oncogenic cell cycle regulating gene and its aberrant activation may be implicated in the pathogenesis associated with *BHD* deficiency (12). Here, we focus on the mechanism for the regulation by folliculin of cyclin D1 expression.

Materials and methods

Cell lines, culture conditions. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS) and antibiotics (penicillin/streptomycin). Treatment (2 h) with wortmannin (final concentration 100 nM), SB203580 (10 μ M), U0126 (10 μ M), rapamycin (20 nM) or SP600125 (20 μ M) was performed using dimethylsulfoxide (DMSO) as the vehicle. Treatment with cycloheximide (10 μ g/ml) was performed using ethanol as the vehicle.

Stable cell lines with an expression vector for folliculin were established from the NR32 (*Bhd*-deficient renal tumor cell line from the Nihon rat) (13). First, NR32 cells were transfected with the plasmid and stable cells were selected in DMEM containing 10% FBS and G418 (final concentration 100 μ g/ml);

Correspondence to: Professor Okio Hino or Dr Toshiyuki Kobayashi, Department of Pathology and Oncology, Juntendo University, School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
E-mail: ohino@juntendo.ac.jp
E-mail: koba396@juntendo.ac.jp

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clones were then selected after limiting dilution and cultured under the same conditions.

Plasmid construction. Rat full length *Bhd* cDNA was cloned into pcDNA3.1 vector (Invitrogen) (4). The *CCND1* promoter region (-1106 to +159) was amplified by PCR from HEK293 cell genomic DNA using the primers PHD1F1 (5'-CCGCTAGC CTCACGCTCAGCAATTCAGT-3') and PHD1R1 (5'-CCAAG CTTATGGCTGGGGCTCTTCCT-3') and subcloned into pGL4.10 vector (Promega, Madison, WI, USA) after digestion with *NheI* and *HindIII*. Fragments of the 3' untranslated region (3'UTR) of human *CCND1*, 3'UTR1 (nt 1-1026) and 3'UTR2 (894-2253), were amplified from HEK293 cell genomic DNA using the following primers: CCNDIUTR1F (5'-GGTCTAGA AGCAGAACATGGACCCCAAG-3') and CCNDIUTR1R (5'-GGTCTAGAGGACTGAAAGTGCTTGAAA-3') for 3'UTR1; CCNDIUTR2F (5'-CCTCTAGAACCTGTTTATG AGATGCTGG-3') and CCNDIUTR2R (5'-CGTCTAGAGCC AAAGCAGGCAGAACCT-3') for 3'UTR2. Amplified products were subcloned into the *XbaI* site (downstream of firefly luciferase coding region) of a vector consisting of pGL4.10 and the *CCND1* promoter. A partial cDNA clone containing the distal 1.8 kb 3'UTR of human *CCND1* (HIBBN77) was obtained from American Type Culture Collection (Manassas, VA, USA). *CCND1* 3'UTR3 (2209-3171) was amplified from HIBBN77 using primers CCNDIUTR3F (5'-CCTCTAGACACAATAC CTCATGCTTAC-3') and CCNDIUTR3R (5'-TTTCTAGAC TTTTCATGTTTGTCTTTTTG-3') and subcloned as 3'UTR1 and -2. A construct containing the full length 3'UTR of *CCND1* was generated from three partial constructs UTR1, -2 and -3 using *PstI* and *NcoI* sites. Full coding sequence of human HuR cDNA was amplified from HeLa cell cDNAs using the primers HuRF1 (5'-CGGAATTCGCCCCGCATCCAGATTTTTGA-3') and HuRR1 (5'-GGCTCGAGTTTGTGGGACTTGTGGT TTT-3') and subcloned into a modified pCAG-GS vector (pCAG-CFLAG) after digestion with *EcoRI* and *XhoI* to introduce a carboxy-terminal FLAG tag (14). A full length cDNA clone (pF1KB9904) for hnRNP L (heterogeneous nuclear ribonucleoprotein L) was obtained from Promega and subcloned into pCAG-CFLAG vector.

RNA interference (RNAi). Transfection of siRNA was performed with Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) at a final concentration of 25 nM. Sequences of siRNAs were described previously (8).

Antibodies. Antibodies against cyclin D1 (C-20 and H295), p27 (C-19), S6K (C-18), HuR (3A2) and hnRNP L (A-11) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-p70 S6K (Thr389), phospho-4E-BP1 (Thr37/46), 4E-BP and acetyl CoA carboxylase were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against histone H1 (AE4) and AUF1 were obtained from Millipore (Billerica, MA, USA). Anti- β -actin (AC15) was obtained from Sigma. The anti-folliculin (C1) antibody has been described previously (4).

Western blot analysis. Protein concentration was determined by the DC-protein assay (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins were subjected to SDS-polyacrylamide gel

electrophoresis and transferred to immobilon-P membranes (Millipore). The membranes were blocked in 1% skim milk in Tris-buffered saline containing 0.05% Tween-20 and then the appropriate antibodies were applied. Rabbit antibodies were detected using the Envision system (Dako, Glostrup, Denmark) as previously described (15). Mouse antibodies were detected using anti-mouse immunoglobulin and streptavidin-biotinylated horseradish peroxidase (GE Healthcare, Buckinghamshire, UK). ECL reagents (GE Healthcare) were used for detection.

RNA isolation, cDNA synthesis and quantitative RT-PCR (qRT-PCR). Total RNA for mRNA analysis was extracted from cultured cells using FastPure RNA kit (Takara Bio, Ohtsu, Japan). cDNA was synthesized using SuperScript II Reverse Transcriptase with random primers (Invitrogen). qRT-PCR was performed using Power SYBR-Green PCR Master mix (Applied Biosystems, Life Technologies, Carlsbad, CA) and the following thermocycler conditions: 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. All values were normalized against β -actin expression levels. Primer sequences were as follows: SBHCD1F1 (5'-TCGGTGTCTACTTCAAATG-3') and SBHCD1R1 (5'-TTCTGTTCCTCGCAGACCTC-3') for *CCND1*; SBHCD3F1 (5'-GCCCTCTGTGCTACAGATT-3') and SBHCD3R1 (5'-CAGTCCACTTCAGTGCCAGT-3') for *CCND3*; (5'-CCATTTGCCATGGTTATAAGG-3') and SBHCER1 (5'-CTTTGCTCGGGCTTTGTCCA-3') for *CCNE1*; SBHVEGFAF1 (5'-CATGAACCTTCTGCTGTCTTGG-3') and SBHVEGFAR1 (5'-ATGATTCTGCCCTCCTCCTT-3') for *VEGFA*; SBHDC25AF1 (5'-CAAGCGTGTCAATTGTTG TGTT-3') and SBHDC25AR1 (5'-CCCTTCAGGACATACA GCTCA-3') for *CDC25A*; SBHBAF1 (5'-CGCGAGAAGATG ACCCAGA-3') and SBHBAR1 (5'-GAGTCCATCACGATG CCAGT-3') for β -actin.

Reporter assay. After 24 h of siRNA treatment, HeLa cells were transfected with the various constructs using Fugene 6 (Roche, Mannheim, Germany). One microgram of each reporter construct was co-transfected with 20 ng renilla luciferase expressing plasmid (pGL4.73, Promega). At 24 h after transfection, the cells were lysed for the analyses. PicaGene dual SeaPangy luminescence kit (Toyo B-net, Tokyo, Japan) was used to measure firefly and renilla luciferase activities.

miRNA analysis. Total RNA for microRNA analysis was isolated using the miRNeasy mini kit (Qiagen, Valencia, CA). cDNA was synthesized using the NCode VILO miRNA cDNA Synthesis kit (Invitrogen). qRT-PCR was performed using Express SYBR-GreenER qPCR SuperMixes and Two-Step qRT-PCR kits (Invitrogen) and the following thermocycler conditions: 50°C for 2 min, 95°C for 10 min and 40 cycles of 95°C for 15 sec and 60°C for 1 min. All values were normalized against U6 expression levels. The primer sequences for U6 and miR-16 amplification were described previously (16). Other primer sequences can be obtained from the NCode miRNA Database provided by Invitrogen (<http://escience.invitrogen.com/ncode/>).

Cell fractionation. Cytoplasmic and nuclear extracts from HeLa cells were obtained using NE-PER nuclear and cytoplasmic extraction reagent (Thermo Scientific, Waltham, MA, USA)

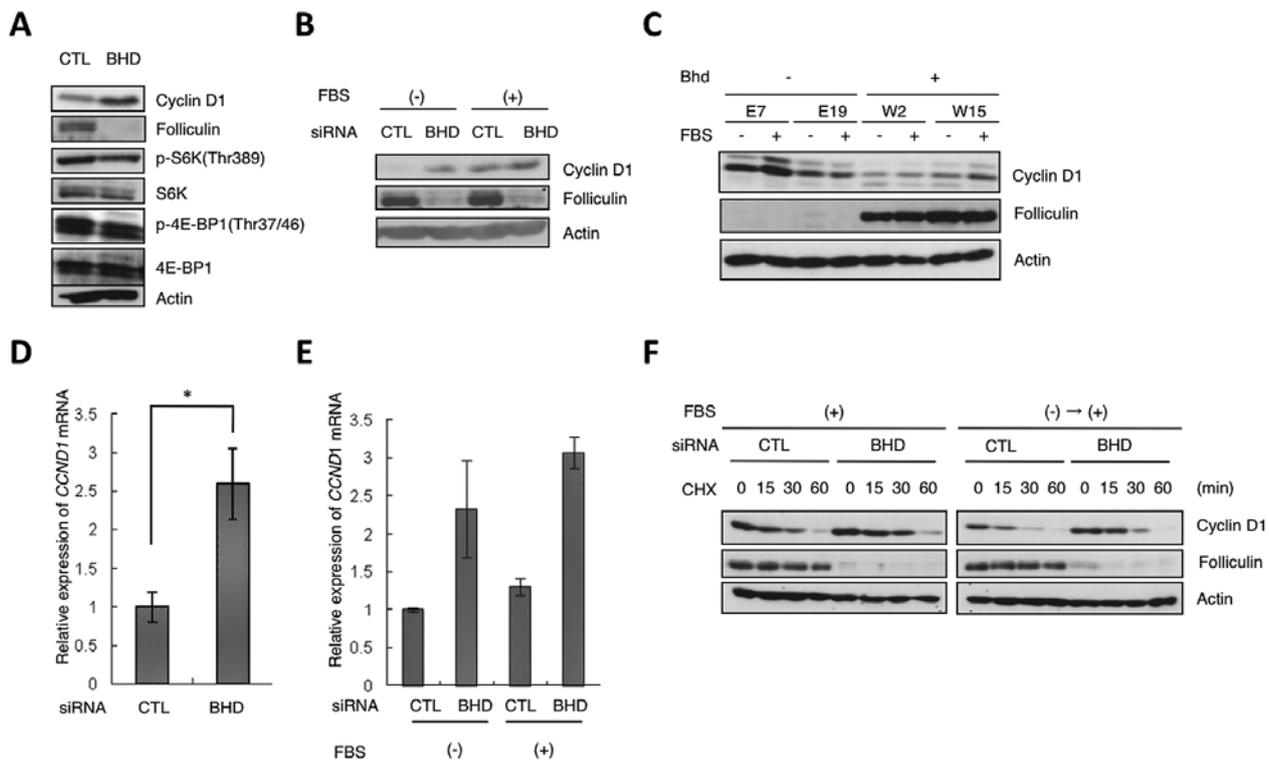


Figure 1. Regulation of cyclin D1 expression by folliculin. (A) Upregulation of cyclin D1 protein expression in HeLa cells by *BHD* knockdown. HeLa cells were transfected with control (CTL) or *BHD* siRNA (BHD). Lysates were analyzed by western blot analysis with the indicated antibodies. β -actin was used as the control. (B) Increased expression of cyclin D1 protein in *BHD* knockdown cells during serum starvation. HeLa cells were transfected as in (A). During the final 24 h of culture, the cells were starved of serum and then cultured in the presence (+) or absence (-) of 10% serum (FBS) for 2 h. Western blot analysis was performed with the indicated antibodies. (C) Expression of cyclin D1 in *Bhd*-deficient (E7, E19) and *Bhd*-restored (W2, W15) cells. FBS (-), serum starvation for 24 h; (+), with serum for 24 h. Lysates were analyzed by western blot analysis with the indicated antibodies. (D) Relative change in *CCND1* mRNA levels induced by *BHD* knockdown. *CCND1* mRNA expression in control HeLa (CTL) and *BHD* knockdown cells (BHD) was analyzed by qRT-PCR. Error bars represent SD; * $P < 0.05$. Three independent experiments were performed in triplicate. (E) qRT-PCR of the relative expression levels of *CCND1* under serum starvation conditions. As in (B), during the last 24 h of the siRNA treatment, the cells were starved of serum and then grown for 2 h in the presence (+) or absence (-) of 10% serum (FBS). Error bars represent SD. Three independent experiments were performed in triplicate. (F) Stability of cyclin D1 protein. HeLa cells were transfected with control (CTL) or *BHD* siRNA (BHD) and cultured under different culture conditions: FBS(+), with serum; FBS(-) \rightarrow (+), cells were arrested by serum starvation for 24 h, and then grown for 2 h in DMEM containing 10% FBS. Cycloheximide (CHX) was added for the indicated time; lysates were analyzed by western blot analysis with the indicated antibodies.

with protease inhibitors (aprotinin, leupeptin, pepstatin A) following the manufacturer's instructions.

Results

Increase in cyclin D1 levels by *BHD* knockdown. siRNA mediated suppression of *BHD* expression in HeLa cells caused an increase in expression of cyclin D1 protein (Fig. 1A). Upregulation of cyclin D1 levels was observed in *BHD* suppressed cells even under serum starvation conditions (Fig. 1B). We examined cyclin D1 protein levels in rat renal tumor cell lines that had been stably transduced with an expression vector for folliculin or an empty vector as a control; the cell lines were derived from NR32, a *Bhd* deficient renal tumor cell line from the Nihon rat. Cyclin D1 levels in the cell lines with restoration of *Bhd* activity (W2 and W15 in Fig. 1C) were lower than in *Bhd* deficient cells (E7 and E19) in both serum starvation and serum added conditions. These results suggest that the downstream pathway of folliculin negatively regulates cyclin D1 expression.

To determine whether the induced increase in cyclin D1 levels after *BHD* knockdown was a result of upregulation of mRNA expression, we performed qRT-PCR. This analysis

showed that *CCND1* mRNA levels in *BHD* knockdown cells were also higher than those in control cells (Fig. 1D and E). We also examined the half-life of cyclin D1 using cycloheximide to ascertain whether cyclin D1 turnover was downregulated by *BHD* knockdown in HeLa cells; no significant difference was present (Fig. 1F). These results suggest that folliculin regulates cyclin D1 expression at transcriptional or posttranscriptional level but not via protein turnover.

***BHD* knockdown does not stimulate *CCND1* promoter activity.** To determine whether folliculin regulates the transcription of *CCND1*, we carried out a reporter analysis by transfecting HeLa cells with a luciferase expressing vector carrying the *CCND1* promoter region (-1106 to +159) (15). Although the *CCND1* mRNA levels increased, luciferase activity was not elevated but rather suppressed in *BHD* knockdown cells (Fig. 2A). Therefore, we concluded that folliculin-mediated control of the level of *CCND1* mRNA is likely not to be via transcriptional mechanisms through known cis-acting elements.

Regulation of *CCND1* mRNA level through 3'UTR by folliculin. Next, we tested whether the expression of *CCND1* was regu-

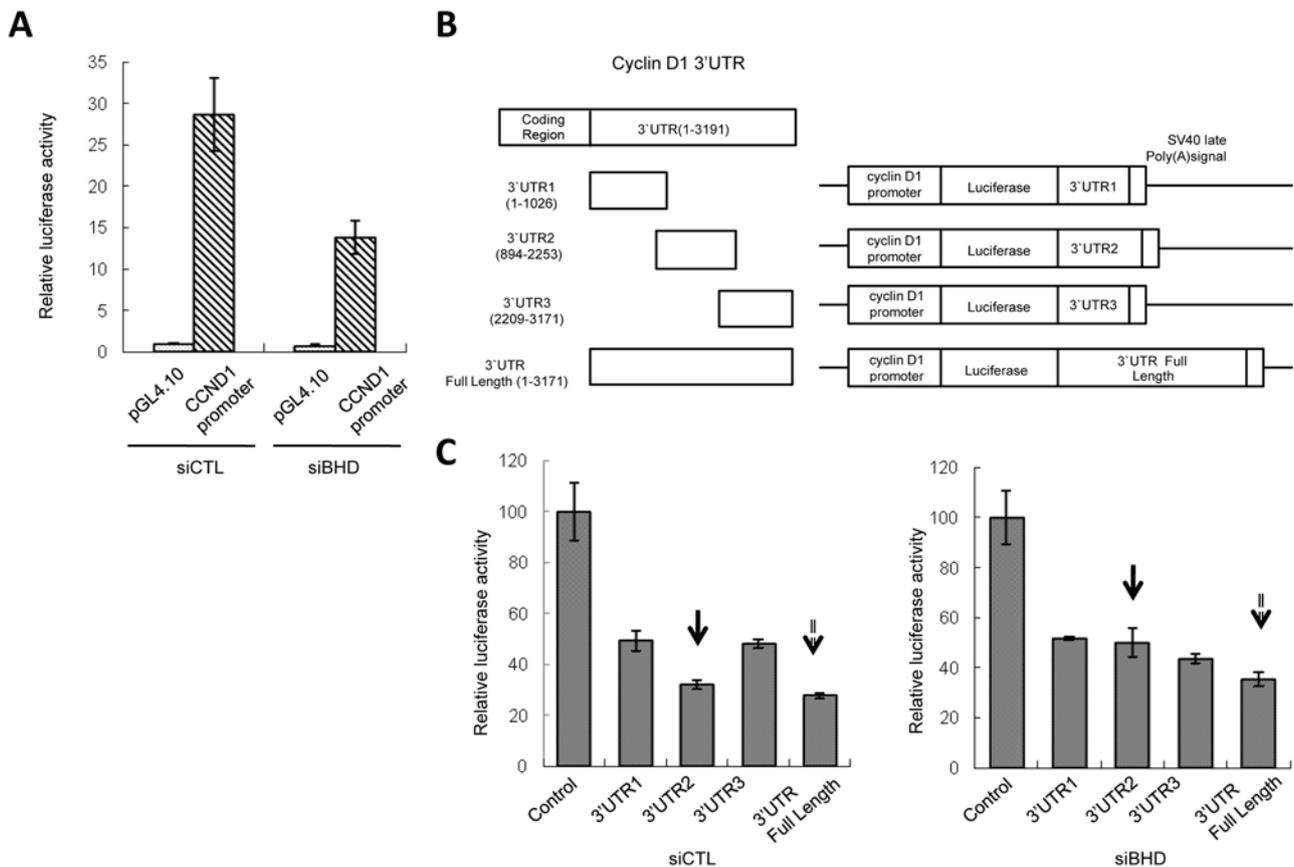


Figure 2. Reporter analyses of *CCND1* promoter and 3'UTR of mRNA. (A) Transcriptional activity of the *CCND1* promoter following *BHD* knockdown. HeLa cells were transfected with control (siCTL) or *BHD* siRNA (siBHD), then transfected with a luciferase expression plasmid carrying the *CCND1* promoter (-1106 to +159) or a pGL4.10 control vector. Lysates were analyzed for luciferase fluorescence to determine the transcriptional activity of the *CCND1* promoter. Three independent experiments were performed in triplicate. (B) Schematic structures of the reporter constructs containing the fragments of the cyclin D1 3'UTR. *CCND1* 3'UTR (3.1 kb) was divided into three overlapping regions and inserted into a luciferase expression vector. (C) Effects of *CCND1* 3'UTR fragments on reporter expression. HeLa cells were transfected with control (siCTL) or *BHD* siRNA (siBHD), then transfected with reporter constructs carrying the *CCND1* 3'UTR fragments or with a control plasmid. Lysates were analyzed for luciferase fluorescence. Error bars represent SD. Three independent experiments were performed in triplicate.

lated by 3'UTR of mRNA. Three constructs carrying different 3'UTR fragments (3'UTR1-3) of *CCND1* (Fig. 2B) exhibited inhibition of reporter activity, possibly due to the existence of negative regulatory elements. Knockdown of *BHD* showed no effect on reporter activity of the constructs containing 3'UTR1 or 3'UTR3. However, for the third construct, 3'UTR2, *BHD* knockdown increased reporter activity (Fig. 2C, arrows with solid line) and this phenomenon was not cancelled by rapamycin treatment (data not shown). Full length *CCND1* 3'UTR also gave similar results, although to a lesser extent than 3'UTR2 (Fig. 2C, arrows with dotted line). We also tested the effects of 3'UTR2 inserted in reverse orientation or upstream of the promoter region. Neither of these constructs elevated luciferase activity in *BHD* knockdown cells compared to control cells, indicating that the function of 3'UTR2 is position and orientation dependent (data not shown). These results imply that the 3'UTR2 sequence in the *CCND1* mRNA influences the level of expression level and that the regulation of *CCND1* by folliculin may be associated with microRNA(s) or RNA binding protein(s) (RBPs) that binds to the 3'UTR2.

CCND1 3'UTR-related microRNAs and *BHD* knockdown. It was reported previously that the 3'UTR of *CCND1* is a

Table I. List of microRNAs predicted to target cyclin D1 by TargetScan.

miR-15a, miR-15b, miR-16, miR-17, miR-19a, miR-19b, miR-20a, miR-20b, miR-34a, miR-93, miR-106a, miR-106b, miR-155, miR-195, miR-302a, miR-302b, miR-302c, miR-302d, miR-424, miR-497, miR-503, miR-519d

target of several microRNAs or RBPs and that these trans-acting factors regulate *CCND1* mRNA stability and/or protein expression (16,18,19). We hypothesized that *BHD* knockdown alters the regulatory pathway that controls microRNA or RBP binding to *CCND1* 3'UTR. We searched for putative microRNA binding sites on *CCND1* 3'UTR using the publicly accessible database TargetScan (<http://www.targetscan.org>) and evaluated the results by comparison with those described in a previous report (Fig. 3) (16). Several of the potential microRNA targets were found to be conserved among vertebrates. We carried out a qRT-PCR on 22 candidate microRNAs (Table I), but did not identify any clear change in their expression levels following *BHD* knockdown (Fig. 4A,

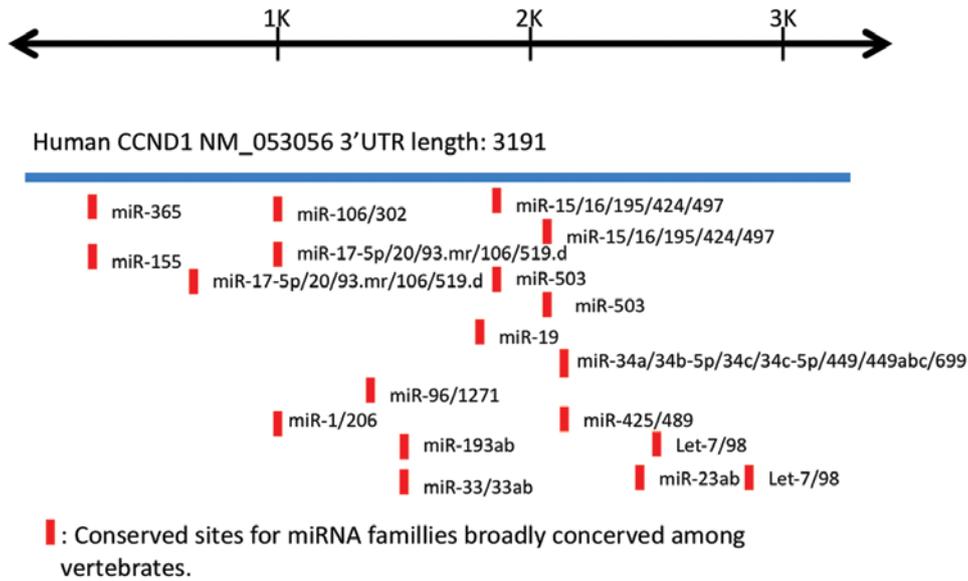


Figure 3. Human *CCND1* 3'UTR and possible miRNA target sites predicted by TargetScan.

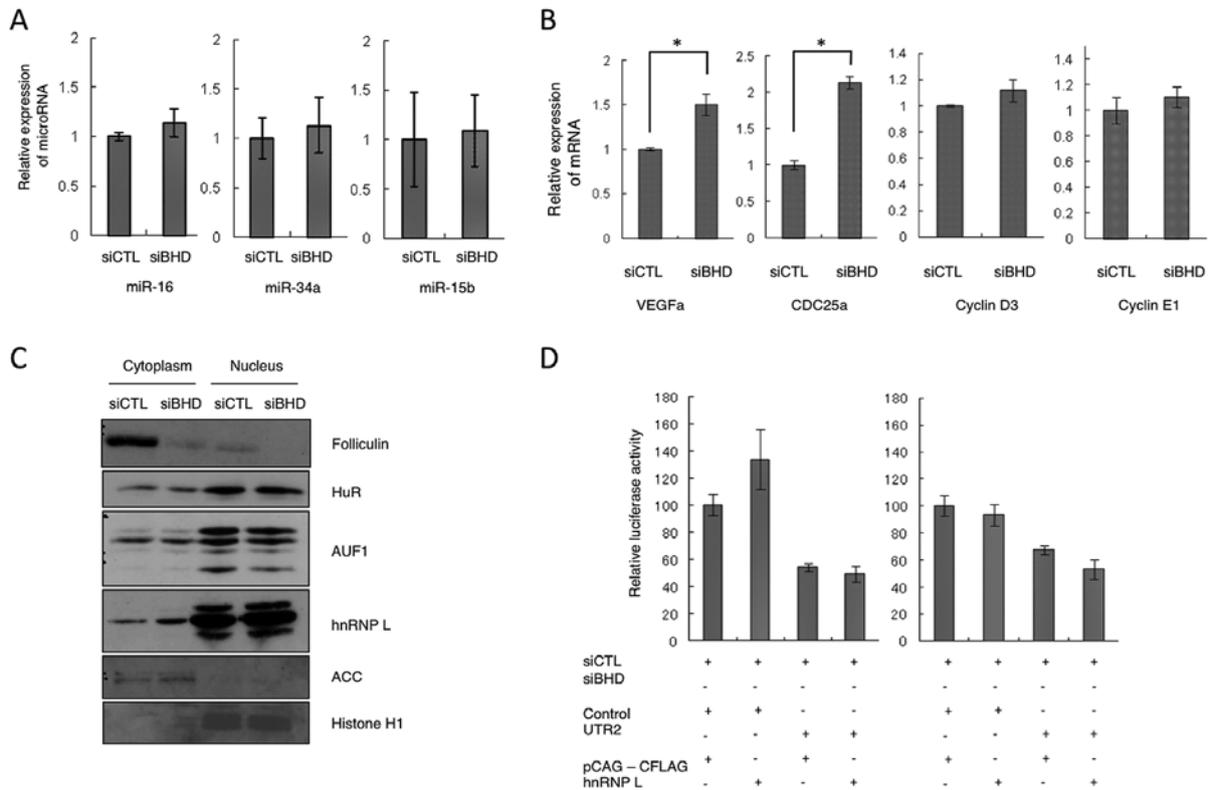


Figure 4. Analyses for microRNAs and 3'UTR binding proteins in *BHD* knockdown cells. HeLa cells were treated with control (siCTL) or *BHD* siRNA (siBHD). (A and B) qRT-PCR analyses for (A) microRNAs that target cyclin D1 mRNA and (B) for genes downstream of miR-16. Error bars represent SD. Three independent experiments were performed in triplicate. *P<0.05. (C) Cell fractionation analysis for 3'UTR binding proteins. Cytoplasmic and nuclear fractions of HeLa cell extracts were analyzed by western blot analysis for HuR, AUF1 and hnRNP L. Acetyl CoA carboxylase (ACC) and histone H1 were analyzed as marker proteins for cytoplasmic and nuclear fractions, respectively. (D) Reporter assay of co-expression of hnRNP L. siRNA-treated cells were co-transfected with a reporter construct (control, promoter only; UTR2, promoter with *CCND1* 3'UTR2) and hnRNP L-expression plasmid or pCAG-CFLAG (empty). Lysates were analyzed for luciferase activity. Error bars represent SD. Two independent experiments were performed in triplicate.

and data not shown). We also investigated the mRNA levels of miR-16 target genes (16,19-21). miR-16 is believed to be a major microRNA that binds to the *CCND1* 3'UTR. The

analysis showed that *BHD* knockdown increased the relative level of expression of *VEGFA*, *CDC25A* mRNA, but not of *CCND3* or *CCNE1* (Fig. 4B). From these observations, we

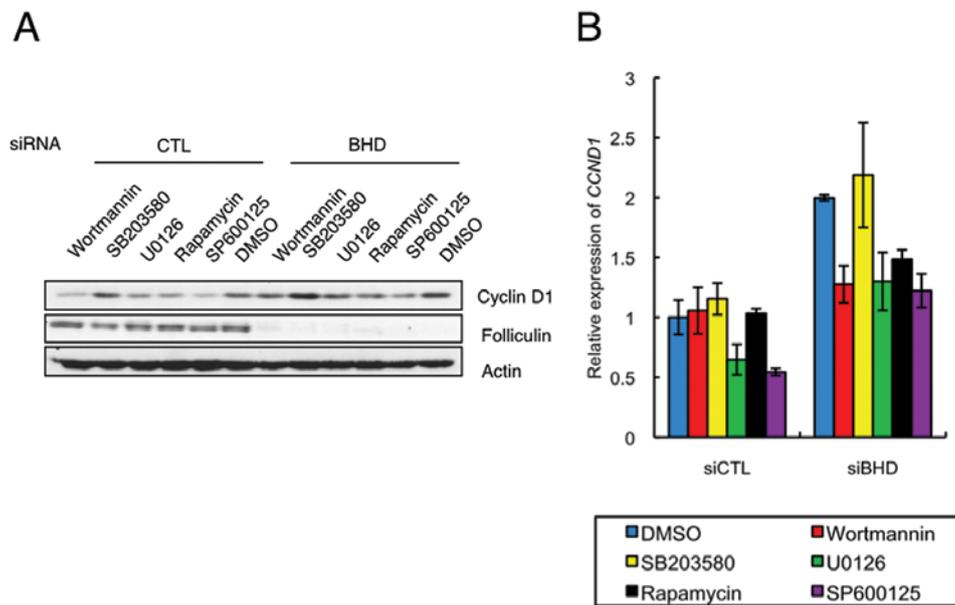


Figure 5. Changes in cyclin D1 protein and mRNA levels induced by inhibitors. (A) HeLa cells were transfected with control (CTL) or *BHD* siRNA (BHD). During the last 24 h, cells were serum-starved and then treated with wortmannin (PI3K inhibitor), SB203580 (p38 inhibitor), U0126 (MEK1/2 inhibitor), rapamycin (mTOR inhibitor), SP600125 (JNK inhibitor) or DMSO for 2 h. Lysates were analyzed by western blot analysis using the indicated antibodies. (B) Relative expression levels of *CCND1* mRNA were compared between control cells (siCTL) and *BHD* knockdown cells (siBHD) treated with the indicated inhibitors by qRT-PCR. Error bars represent SD. Three independent experiments were performed in triplicate.

conclude that the microRNAs listed here do not play a major role in cyclin D1 regulation by folliculin.

RNA binding proteins and *BHD* knockdown. We analyzed three RBPs, HuR, AUF1 and hnRNP L, that have previously been reported to bind to 3'UTR and to control mRNA stability. HuR and AUF1 have been identified as binding to the AU rich element (ARE) on the *CCND1* 3'UTR. HuR stabilizes *CCND1* mRNA, whereas AUF1 causes its destabilization (18). hnRNP L has been reported to bind to the CA rich element of *VEGFA* and to increase mRNA stability (22). First we performed a cell fractionation analysis and found that cytoplasmic hnRNP L levels were increased by *BHD* knockdown (Fig. 4C), whereas those of HuR and AUF1 were unchanged in both the cytoplasm and nucleus. To determine whether these RBPs could bind *CCND1* 3'UTR and regulate mRNA expression in a folliculin-dependent manner, a reporter assay using constructs expressing hnRNP L and HuR was performed. We found that luciferase activities from the 3'UTR2-containing reporter were not elevated following co-transfection of hnRNP L or HuR, regardless of the folliculin status (Fig. 4D and data not shown). Accordingly, although possible regulation of hnRNP L localization was suggested, the folliculin-dependent association between 3'UTR2 and RBPs could not be identified in our experiments.

***BHD* knockdown elevates rapamycin sensitivity of *CCND1* mRNA expression.** To explore the signaling pathway modulated after *BHD* knockdown, we investigated the effects of several signal inhibitors on cyclin D1 expression. This analysis showed that 2-h treatment of wortmannin, U0126, rapamycin or SP600125 inhibited cyclin D1 protein levels in control and *BHD* knockdown cells, whereas SB203580 was ineffective in

both cell types (Fig. 5A). Each of the positive inhibitors had a similar effect in control and *BHD* knockdown cells relative to the vehicle control. Interestingly, the relative levels of *CCND1* mRNA in drug treated *BHD* knockdown cells differed from that in control cells (Fig. 5B). Treatment with wortmannin or rapamycin significantly decreased the relative levels of *CCND1* mRNA in a *BHD* knockdown-specific manner (Fig. 5B). These results suggest that the regulatory mechanism for *CCND1* mRNA expression was more sensitive to inhibition of the mTORC1 pathway in *BHD* knockdown cells.

Discussion

Although *BHD* is regarded as a tumor suppressor gene, its function is not clearly understood and its contribution to cell cycle regulation, including the suppression of cyclin D1, has yet to be determined. In this study, we found an increase in cyclin D1 protein and mRNA levels in *BHD* knockdown cells. Based on the results of our experiments, we suggest that the increase in *CCND1* mRNA and protein levels induced by *BHD* suppression is mediated, at least in part, by a 3'UTR (3'UTR2)-related mechanism.

The *CCND1* promoter region (-1106 to +159) used in this study was reported to contain core positive regulatory elements that respond to cell cycle promoting signals (17). Although we did not identify any element in the *CCND1* promoter region that responded to *BHD* downregulation, the existence of such cis-acting elements in other regions of the *CCND1* gene cannot be excluded.

One of the mechanisms for regulation of mRNA levels is microRNA-mediated degradation (23). There are a number of microRNA binding sites in the *CCND1* 3'UTR2 and many

of these are conserved between humans and rat (14). For example, miR-16 has been implicated in the regulation of cell cycle related genes including cyclin D1 (16,19-21). The increase in *CCND1* mRNA levels after knockdown of *BHD* could be explained as the consequence of downregulation by a microRNA-mediated mechanism. To date, we have not identified any expression change in microRNAs nor of miR-16 target genes in *BHD* knockdown cells. However, as there are additional microRNA binding sites to those analyzed in 3'UTR2, the potential contribution of other microRNAs remains to be determined. Possibly, comprehensive microRNA analysis will identify candidates for 3'UTR2-associated miRNAs downstream of folliculin.

The *CCND1* 3'UTR has been reported to have seven AREs composed of an AUUUA pentamer; six of these seven motifs are present in 3'UTR2 (24). Many RBPs have been found to selectively recognize and bind to the ARE of mRNAs and to modulate the translation and/or stability of the mRNAs (25). We tested the effects of three RBPs (HuR, AUF1, hnRNP L) on 3'UTR2-mediated regulation, but did not find a relationship between these RBPs and folliculin. Other RBPs may bind *CCND1* mRNA 3'UTR2 to regulate folliculin-mediated mRNA translation/stability. Interestingly, cytoplasmic hnRNP L levels were increased by *BHD* knockdown in our study. hnRNP L has been reported to bind to CA repeats/CA rich elements in the 3'UTR of *VEGFA*, *BCL-2* and *GLUT1*, and to increase the stability of their mRNAs (22,26,27). Notably, the *CCND1* 3'UTR2 also has CA repeats. Jafarifar *et al* reported that hypoxia induces translocation of nuclear hnRNP L to the cytoplasm, resulting in a marked increase in hnRNP L binding to *VEGFA* mRNA and in the stability of *VEGFA* mRNA (22). Although our reporter analysis did not detect any specific effects, it is possible that in *CCND1* 3'UTRs other than 3'UTR2, some cooperative elements are required for the efficient binding and regulation by hnRNP L. Singh *et al* (28) suggested that folliculin may be a component of an RBP complex on the basis of observation that the *Drosophila* folliculin protein binds to RBP9, a homologue of human Hu protein, and shows weak homology to Pumilio, another RBP (29). Thus, the possibility of direct binding and regulation of *CCND1* mRNA 3'UTR2 by a folliculin-RBP complex still remains.

We performed a search for signaling molecules involved in the upregulation of cyclin D1 expression using various signal inhibitors. This analysis showed that rapamycin and wortmannin suppressed *CCND1* mRNA levels in *BHD* knockdown cells but not in control cells. These results suggest that the mTORC1 pathway may contribute to upregulation of *CCND1* expression caused by *BHD* knockdown. Rapamycin has been reported to affect *CCND1* mRNA and protein stability and thereby decrease levels of cyclin D1 protein (30). Recent studies have also reported that rapamycin regulates *CCND1* transcription and mRNA stability in an AKT-dependent manner; under conditions of relatively quiescent AKT activity, treatment of cells with rapamycin results in the upregulation of *CCND1* transcription and mRNA stability, whereas in cells containing active AKT, rapamycin these changes are repressed (31,32). This phenomenon may be related to the rapamycin sensitivity of *BHD* knockdown cells found in this study. We demonstrated that S6K1 phosphorylation and total mTOR levels are decreased by *BHD* knockdown in HeLa cells, and this also may lead to

an increased sensitivity to the effects of rapamycin and wortmannin (8). Cyclin D1 protein and mRNA levels were decreased by rapamycin treatment in *BHD* knockdown cells, but this treatment did not affect the upregulated reporter activity by 3'UTR2 in these cells. Thus, although mTORC1 signaling is required, it may be not a direct cause of the *CCND1* mRNA increase caused by *BHD* downregulation.

In recent studies, it has been reported that cyclin D1 protein levels were elevated in renal cell tumors of *Bhd* heterozygous knockout mice and the absence of folliculin resulted in an increase in *CCND1* mRNA levels in UOK257 cells (10,11). Analysis of the folliculin-mediated mechanism regulating cyclin D1 expression may provide important clues to understanding the pathogenesis of BHDS and other *BHD* mutation-associated diseases.

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