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ORIGINAL ARTICLE

# Transgenic rescue from embryonic lethality and renal carcinogenesis in the Nihon rat model by introduction of a wild-type *Bhd* gene

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**We recently reported that a germline insertion of a single nucleotide in the rat homologue of the human Birt–Hogg–Dubé gene (*BHD*) gives rise to dominantly inherited cancer in the Nihon rat model. In this study, we constructed transgenic Nihon rats with introduction of a wild-type *Bhd* gene to ascertain whether suppression of the Nihon phenotype is possible. Rescue from embryonic lethality of mutant homozygotes (Nihon/Nihon) and suppression of renal carcinogenesis in heterozygotes (Nihon/+) were both observed, defining the germline *Bhd* mutation in the Nihon rat as an embryonal lethal and tumor predisposing mutation. This transgenic rescue system will be useful to analyse *Bhd* gene function, its relation to tumorigenesis *in vivo*, and genetic–environmental interactions in carcinogenesis.**
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**Keywords:** renal carcinoma; *Bhd* gene; Nihon rat; transgenic rescue

## Introduction

Hereditary cancer was described in the rat by Eker and Mossige in Oslo in 1954 (Eker and Mossige, 1961). The Eker rat model of hereditary renal carcinoma (RC) was the earliest example of a Mendelian dominantly inherited predisposition to a specific cancer in an experimental animal (Hino *et al*., 1993). We and others identified a germline mutation in the rat homologue of the human tuberous sclerosis gene (*TSC 2*) as the predisposing Eker gene (Hino *et al*., 1994; Yeung *et al*., 1994; Kobayashi *et al*., 1995). Recently, we found a novel hereditary RC model in the Sprague–Dawley (SD) rat in Japan in 2000. We have named this RC model the 'Nihon' rat (Okimoto *et al*., 2000). The Nihon rat is also an example of a Mendelian dominantly inherited predisposition for development of RCs, which are predominantly of the clear cell type, and carries a single gene mutation like that in the Eker rat. The transmission mode of the Nihon rat gene revealed that the homozygous mutant condition induce embryonic lethality by mid-gestation. In heterozygotes, RCs develop from early preneoplastic lesions, seen as early as 3 weeks of age, into adenomas by 8 weeks of age, and penetrance for this RC gene was virtually complete (Okimoto *et al*., 2004a).

The Nihon gene is located on the distal part of the rat chromosome 10 (Hino *et al*., 2001). Recently, we identified a germline mutation in the rat *BHD* homologue that predispose to the renal cancer phenotype in the Nihon rat. Sequence analysis revealed that there was an insertion of a cytosine (C) in a C tract within exon 3. This germline mutation results in a frameshift and produces a stop codon 26 amino-acids downstream (Okimoto *et al*., 2004b).

The detection of loss of the wild-type allele supports the hypothesis that a second somatic mutation is the rate-limiting step for renal carcinogenesis in the Nihon rat model, suggesting that it is a novel tumor-suppressor gene fitting Knudson's two-hit model.

The Birt–Hogg–Dubé (BHD) syndrome, originally described by Birt, Hogg, and Dubé in 1977, is an autosomal dominant genetic disease characterized by hair follicle hamartomas (fibrofolliculomas) and spontaneous pneumothorax, lung cysts and RCs (Birt *et al*., 1977). The recent discovery of the human *BHD* gene is an important first step toward understanding the mechanism of tumorigenesis in BHD patients; however, the function of the BHD product (folliculin) remains to be elucidated (Nickerson *et al*., 2002). The phenotype in humans differs from that in the Nihon rat, except for the occurrence of RCs. Given the lack of knowledge regarding the molecular mechanism underlying human BHD, the potential of the Nihon rat for contributing to elucidation of the *BHD*/*Bhd* gene's role in renal carcinogenesis, as well as for studying species-specific differences in tumorigenesis and/or cell-type-specific carcinogenesis deserved attention.

In this study, to confirm that a tumor predisposition in the Nihon rat is caused by the *Bhd* germline mutation and to establish an *in vivo* system for analysis of the *Bhd* gene's role, we constructed transgenic Nihon rats by introduction of a chimeric minigene consisting of a *Bhd* cDNA and its 5′-upstream promoter region.

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## Results and Discussion

*Establishment of transgenic Nihon rats carrying wild-type* Bhd *transgene*

We reported that rat *Bhd* gene consists of 13 exons and shows CpG island-like property near exon 1 (Okimoto *et al*., 2004b). In addition, we detected promoter activity in a 0.75 kb 5′-upstream sequence in cultured cells by reporter assay (our unpublished observation). Therefore, we initially chose the 2.3 kb 5′-upstream region including this 0.75 kb sequence as a regulatory region for the expression of transgene (Tg). We constructed a wild-type rat *Bhd* Tg consisting of a genomic DNA fragment including the rat *Bhd* promoter and intron 1, a *Bhd* cDNA of exon 2–13 and the poly(A) addition signal sequence (Figure 1a). After injection of the Tg fragment into eggs obtained from Nihon rat/wild-type SD rat mating, offspring were examined for the retention of Tg DNA (Figure 1b). In Southern blot analysis, ~8.5, ~7 and ~6 kb *Bam*HI fragments were detected as endogenous *Bhd* gene by the probe employed. In addition of these bands, a Tg-specific band with the expected ~4 kb size was detected. Totally, we obtained seven transgenic founders. Of these, three rats were heterozygous mutants for endogenous *Bhd*. Germline transmission was examined and expression of mRNA from Tgs were analysed by Northern blot analysis (Figure 1c). In brain and kidney, expression of Tg-specific mRNA was detected. We selected two lines (2L and 6L) for further analyses. Copy numbers of Tg in 2L and 6L were estimated as ~7 copies and ~4 copies, respectively, by Southern blot analysis (Figure 1d).

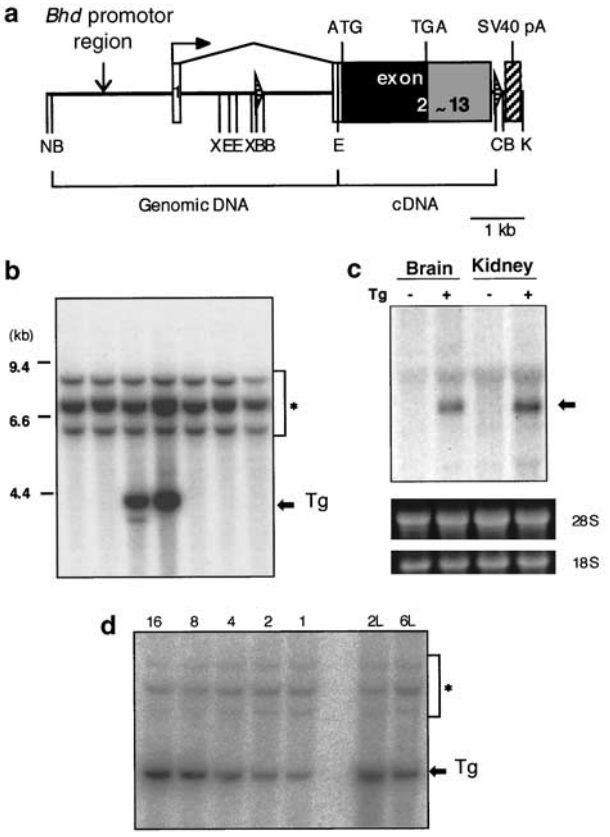


**Figure 1** Generation of the *Bhd* transgenic founders. (**a**) Structure of the wild-type *Bhd* Tg used in this study. Black and shaded regions indicate coding and noncoding regions in the cDNA, respectively. The positions of translational start (ATG) and stop (TGA) codons are shown above. The striped box denotes a fragment from SV40 DNA containing poly A signals (SV40 pA). N; *Not*I, B; *Bam*HI, X; *Xho*I, E; *Eco*RI, C; *Cla*I, K; *Kpn*I. Two loxP sequences are shown by triangles. (**b**) Southern blot analysis of the transgenic founders. DNA samples were digested with *Bam*HI and probed with a $^{32}$P-labeled rat *Bhd* cDNA fragment covering exons 4–12. Representative result is shown. Arrow and asterisk indicate the bands of Tg and endogenous *Bhd* gene, respectively. Positions of size markers ($\lambda$/*Hin*dIII) are shown on the left side. (**c**) Northern blot analysis of the Tg expression. Total RNAs from the brain and kidney were probed with SV40 pA probe. Results of non-Tg-carrying rat (−) and Tg-carrying rat (+) are shown. Arrow indicates the band of mRNA from Tg (~3 kb). 18S and 28S ribosomal RNA are shown below. (**d**) Estimation of copy numbers in line 2L and 6L. Southern blot analysis was performed with a plasmid standard. Before *Bam*HI digestion, DNA from a control non-transgenic rat was mixed with Tg plasmid with different amounts to make standard samples. Numbers above standard lanes indicate calculated copy numbers of plasmid as in the genomic DNA. Copy numbers in 2L and 6L were estimated as ~7 copies and ~4 copies by comparison with standard samples. Arrow and asterisk indicate the bands of Tg and endogenous *Bhd* gene, respectively.

*Suppression of renal carcinogenesis by wild-type* Bhd *Tg in the Nihon rat*

We investigated the effect of extra copies of the wild-type *Bhd* gene on renal carcinogenesis in heterozygous *Bhd* mutants of the Nihon rat. At first, we killed Tg-carrying heterozygous mutants and control non-Tg-carrying heterozygous mutants at 22 weeks of age and compared the kidneys of those rats. In control Nihon rats, multiple renal tumors had developed at this stage (Figure 2 and data not shown). On the other hand, kidneys from Tg-carrying heterozygous mutants were completely free from renal tumors macroscopically and microscopically (Figure 2 and data not shown). A total of 18 Tg-carrying heterozygous mutants were analysed between the ages of 22 and 54 weeks and none of those rats showed tumors in the kidney, whereas all control rats ($n=22$) developed tumors (Table 1). These results clearly show that the extra copies of wild-type *Bhd* gene suppress renal carcinogenesis in the Nihon rat by preventing complete loss of *Bhd* function. Thus, the germline *Bhd* mutation appeared to be a bona fide cause of hereditary renal carcinogenesis in the Nihon rat (Okimoto *et al*., 2004b).

*Rescue of homozygous* Bhd *mutants from embryonic lethality by wild-type* Bhd *Tg*

We also examined whether extra copies of wild-type *Bhd* gene could function during the embryonic stage and prevent the lethality of homozygous *Bhd* mutant in the Nihon rat. Tg-carrying heterozygous mutants and non-Tg-carrying heterozygous mutants were mated to generate *Bhd* homozygous mutants. By Southern blot analysis of offspring, it was apparent that *Bhd* homozygous mutants were born (Figure 3a). DNA was also analysed by PCR to ascertain the endogenous exon3

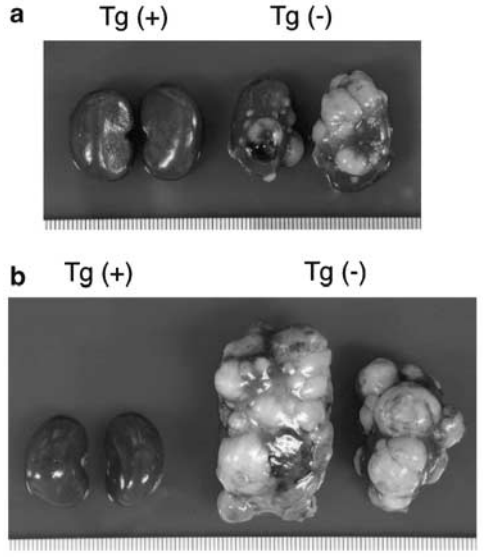


**Figure 2** Suppression of renal carcinogenesis in heterozygous *Bhd* mutants by introduction of the wild-type *Bhd* Tg. (**a**) Kidneys from male heterozygous *Bhd* mutants of line 2L at 22 weeks of age. (**b**) Kidneys from male heterozygous mutants of line 6L at 46 weeks of age. Tg (+): Tg-carrying rats, Tg (−): non-Tg-carrying rats.

**Table 1** Number of offspring of Tg Nihon rat

| | *Genotype of* Bhd | | | | | |
|---|---|---|---|---|---|---|
| | +/+ | | +/− | | −/− | |
| | Tg(+) | Tg (−) | Tg(+) | Tg (−) | Tg(+) | Tg (−) |
| Line 2L | 12 | 7 | 16 | 17 | 8 | 0 |
| Line 6L | 1 | 1 | 2 | 5 | 2 | 0 |
| Total | 13 | 8 | 18 | 22 | 10 | 0 |

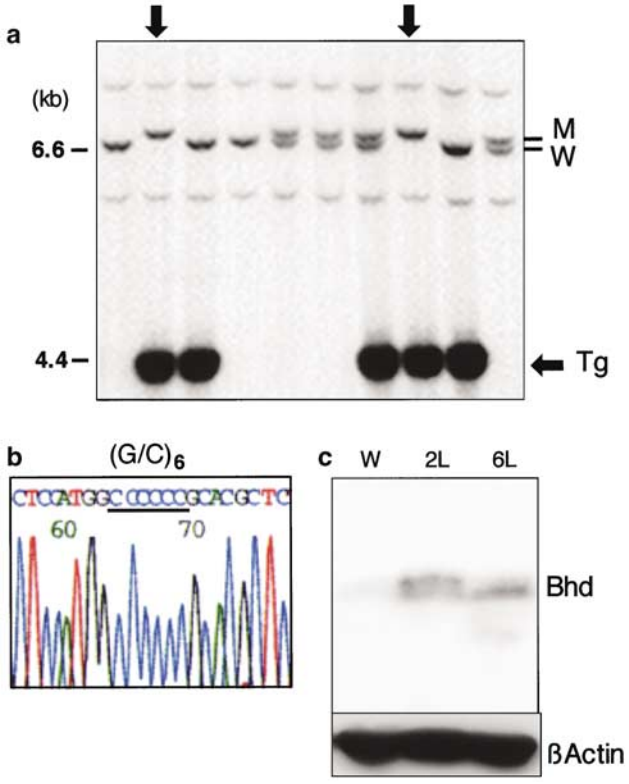


**Figure 3** Rescue of *Bhd* homozygous mutants from embryonic lethality by introduction of the wild-type *Bhd* Tg. (**a**) Genotyping of offspring from a double heterozygous breeding with Tg. Southern blot analysis was performed with *Bam*HI digestion as in Figure 1. Positions of germline mutant-associated band (M), wild-type band (W) and Tg (arrow) are shown on the right side. Vertical arrows above show rescued *Bhd* homozygous mutants. (**b**) Sequence analysis of germline mutation in rescued homozygous mutants. PCR-amplified exon 3 fragment of endogenous *Bhd* gene was directly sequenced. The result of a rescued homozygous mutant is shown. Homozygosity of six G/C repeats (underline), which was created by a germline mutation, is shown. (**c**) Expression of folliculin in rescued homozygous mutants. Kidney protein extracts from wild-type (W) and rescued homozygous mutant rats from line 2L (2L) and line 6L (6L) were analysed by Western blot. Upper panel shows the expression of Bhd protein and lower panel shows control $\beta$-actin. Rescued homozygous mutants shows much higher expression of Bhd protein compared with wild-type rat.

sequence. We could then confirm that a homozygous one base insertion (germline mutation) was present in the G/C tract in exon3 (Figure 3b). All *Bhd* homozygous mutants carrried wild-type *Bhd* Tgs (Table 1). No homozygous mutants without Tg were born. The results were confirmed in two independent lines. These results clearly indicate that the wild-type *Bhd* Tg functioned during embryonic development and rescued homozygous *Bhd* mutants from the lethality. In renal tumor cells from Nihon rat, which lost the wild-type *Bhd* allele, no Bhd protein (folliculin) was detected although mRNA from the mutant *Bhd* allele was significantly expressed (Okimoto *et al*., 2004b). This indicates that the mutant allele does not produce folliculin. We confirmed the expression of folliculin in rescued homozygous *Bhd* mutants (Figure 3c). In kidneys of homozygous mutants from both lines, folliculin was detected with higher amount compared with wild-type control. According to the differnce in copy number of Tg, line 2L showed higher expression than line 6L. So far, we have not detected apparent phenotypic difference between these two lines although further detailed examination is needed.

### *Development of renal tumors in rescued homozygous* Bhd *mutants*

Interestingly, in contrast to the situation in Tg-carrying heterozygous *Bhd* mutants, renal carcinogenesis was observed in rescued homozygous mutants during long-term observation (Figure 4a). By the age of 22–49 weeks, all homozygous mutants developed RCs. These RCs showed the clear cell-type histology that is also observed in heterozygous mutants of the Nihon rat (Figure 4b). As the expression of enough amount of folliculin from Tg was detected in the kidney by Western blot analysis (Figure 3c), we speculated that Tg was lost

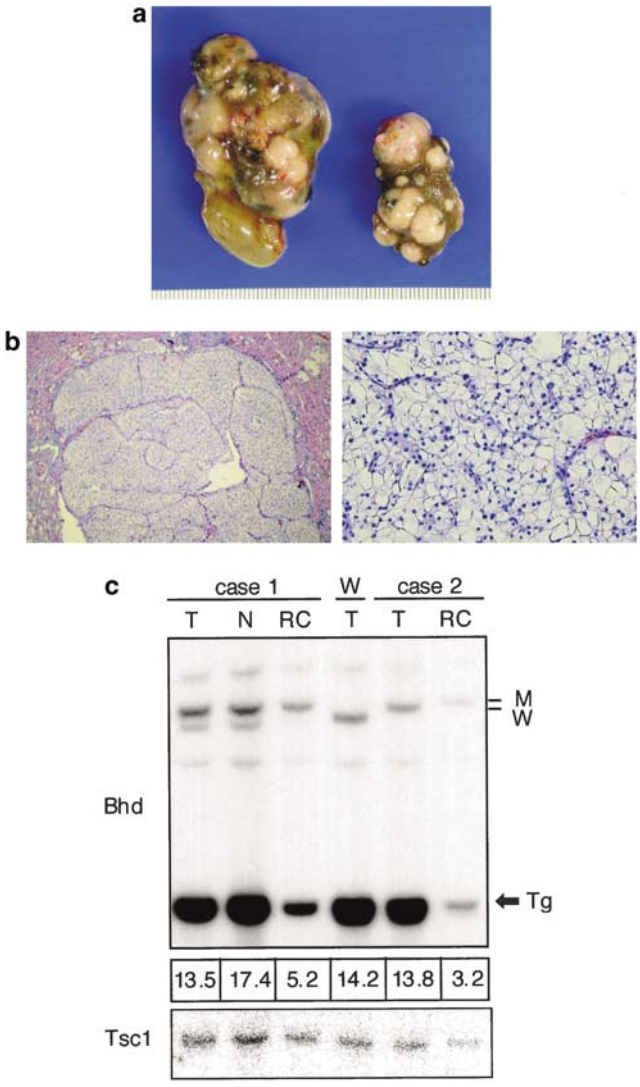


**Figure 4** Renal carcinogenesis observed in rescued *Bhd* homozygous mutants. (**a**) Kidneys from a rescued male *Bhd* homozygous mutant (line 6L) at the age of 46 weeks. They show multiple and bilateral tumors. (**b**) A histology of clear cell renal carcinoma (RC) in the rescued homozygous mutant. The right panel shows an enlarged view of a part of the carcinoma in the left panel. (**c**) Loss of Tg in RCs in the rescued homozygous mutants. Results of two rescued homozygous mutants (cases 1 and 2) and a Tg-carrying wild-type rat (W) are shown. DNAs from tail (T), normal part of kidney (N) and RC were digested with *Bam*HI and subjected to Southern blot analysis. Upper panel shows the results of hybridization with *Bhd* probe. Positions of germline mutant-associated band (M), wild-type band (W) and Tg (arrow) are shown on the right side. The ratios of Tg band to W or M bands (defined as 1), which were calculated after measuring radioactivity, are shown below. Lower panel shows the result of *Tsc1* probe to check the loading difference. Another *Niban* probe was also used for loading check and similar result was obtained (data not shown). These conrol genes are localized on different chromosomes.

or suppressed at the origination of carcinogenesis. Indeed, loss of Tg was detected in those RCs compared with a normal part of kidney or tail by Southern blot analysis (Figure 4c). Although multicopy Tgs were introduced by transgenic technique, they could be inserted in single sites on the genome with tandem arrangement. It is not plausible that multiple *Bhd* Tgs were simultaneously or sequentially inactivated by point mutations. Accordingly, it is conceivable that, at least in part, the simultaneous loss of multicopy Tgs by chromosome loss or other mechanisms caused renal carcinogenesis in rescued homozygous mutants, like a second hit on the wild-type *Bhd* allele in heterozygous mutants. However, suppression of the expression from Tgs by some epigenetic regulation should not be excluded as a cousative mechanism. We have observed similar tumor development and loss of Tg in homozygous *Tsc2* mutant rats rescued by wild-type *Tsc2* Tg (unpublished results).

The Nihon rat is a useful and valuable animal model of renal carcinogenesis and can contribute to understanding human renal carcinogenesis. As our use of the transgenic Eker rat system for functional analysis of the Tsc2 gene product (Kobayashi *et al*., 1997; Momose *et al*., 2002), the transgenic Nihon rat will be a useful system for functional analysis of folliculin *in vivo*. In addtion, we are now establishing cultured embryonic fibroblast cells derived from homozygous *Bhd* mutants carrying Tg. As loxP sequences were introduced in Tg DNA, *in vitro* conditional ablation of Tg by Cre-mediated recombination might be possible. This conditional ablation will become another useful experimental system for the elucidation of folliculin's function.

## Materials and methods

*Construction of a wild-type* Bhd *Tg*

To screen a rat genomic DNA library (cosmid), a genomic DNA fragment containing the 5′-upstream region of the rat *Bhd* gene was amplified by PCR. The primers used were BPR-F1 (5′-ACAAGGGCCCATGCTATGC-3′) and BPR-R1 (5′-CGGAAGCTTCTCGCAACTG-3′) designed after searching the rat HTGS genomic database (NCBI) with the rat *Bhd* cDNA sequence (Okimoto *et al*., 2004b). By using this PCR fragment as a probe, a cosmid clone (cos-rBhd1) covering the region from 2.3 kb 5′-upstream region to exon 2 of *Bhd* was isolated. By serial cloning steps, a plasmid for wild-type *Bhd* Tg (pB-rBhd-Tg) was constructed (Figure 1a). Briefly, Tg consisted of a 5.4 kb genomic DNA fragment (from 2.3 kb 5′-upstream to an *Eco*RI site in exon 2) and a 3 kb cDNA fragment (covering all of the 3′ region from the *EcoRI* site in exon 2) and 0.2 kb fragment containing the SV40 poly A signal (Kobayashi *et al*., 1997). It contained also two loxP sequences in intron 1 and the 3′-noncoding region in the same orientation. For microinjection, the Tg fragment was purified after digestion of pB-rBhd-Tg with *Not* I and *Kpn*I.

*Generation of transgenic founder rats and establishment of lines*

Male Nihon rats on SD background were mated with female wild-type SD rats and fertilized eggs were obtained. The Tg was introduced into eggs by microinjection according to standard protocol (Kobayashi *et al*., 1997). Transgenic founders heterozygous for the germline *Bhd* mutation were mated with Nihon rats or wild-type SD rats. Transgenic founders without germline *Bhd* mutation were mated with Nihon rats. All subsequent crossings were carried out on SD background.

*DNA isolation and Southern blot analysis and PCR*
Genomic DNAs were isolated from rat tails by digestion with proteinase K and extraction with phenol/chloroform as described previously (Kobayashi *et al*., 1995). For Southern blot analysis, 10 $\mu$g of each DNA was digested with *Bam*HI and separated by 1% agarose gel electrophoresis and transferred onto a nylon membrane (Biodyne B; Pall) under alkaline conditions (0.4 N NaOH). Prehybridization, hybridization and washing were performed as described (Kobayashi *et al*., 1995). The probe used were as follows, Rat *Bhd*: a 1.2 kb *Eco*RI-*Xho*I cDNA fragment covering exons 4–12, Rat *Tsc1*: a 1.7 kb *Eco*RI fragment from full-length cDNA (Satake *et al*., 1999), Rat *Niban*: a 3.1 kb cDNA fragment (Majima *et al*., 2000). In Southern blot analysis for copy number estimation, pB-rBhd-Tg DNA was used as a standard and mixed with genomic DNA of non-transgenic rat. For copy number estimation and loss of Tg analysis, the Fuji BAS2500 image analyzer (Fuji Film) was used to measure radioactivity.

For genotyping of endogenous *Bhd* by PCR, the following primer sets were used. RBE4-F1 (5′-GATGCCGTCCTTCCC TACAG-3′, forward in intron 2) and RBE4-R1 (5′-AAGGC TAAAACCAGCTCACG-3′, reverse in intron 3) to amplify exon 3. PCR-amplified fragments were subjected to direct sequence analysis.

*RNA isolation, Northern blot analysis*
Total RNAs were isolated from tissues using TRIzol Reagent (Invitrogen life technologies). For Northern blot analysis, 10 $\mu$g of total RNAs were electrophoresed through a denaturing 1% agarose gel containing 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA and 6% formaldehyde. Then, RNAs were then transferred onto a nylon membrane and hybridized with probes (Kobayashi *et al*., 1995).

*Western blot analysis*
Total proteins from the kidney were obtained by homogenization of tissue samples in SDS–PAGE gel loading buffer and protein concentration was determined by DC-protein assay (Bio-Rad). Equal amount of proteins were subjected to SDS–PAGE and transferred onto Immobilon-P membrane (Millipore). Immunoblot was performed using a rabbit polyclonal antibody for folliculin (Okimoto *et al*., 2004b) and a mouse monoclonal antibody for $\beta$-actin (AC15, Sigma). ECL reagents (Amersham) were used for detection step.

*Histological analysis*
Sections from kidneys were stained with hematoxylin and eosin and were examined microscopically.

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