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Transforming Growth Factor Alpha (TGF- α) and Other Targets of Tumor Necrosis Factor-Alpha Converting Enzyme (TACE) in Murine Polycystic Kidney Disease

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Abstract

Transforming growth factor-alpha (TGF- α) is abnormally expressed in autosomal recessive polycystic kidney disease (ARPKD). Tumor necrosis factor-alpha converting enzyme (TACE), a metalloproteinase, mediates TGF- α processing. In this study, we sought to determine whether TGF- α was an absolute requirement for renal cystogenesis and whether its absence would modulate disease severity or related growth factors/receptors expression. *Bpk* heterozygotes were bred with TGF- α null mice to produce cystic and noncystic offspring with or without TGF- α . Assessments included kidney weight (KW), body weight (BW), blood urea nitrogen (BUN), and kidney and liver immunohistology. Western analysis assessed kidney expression of amphiregulin (AR), epidermal growth factor (EGF), heparin-binding EGF (HB-EGF), and their receptors, EGFR and ErbB4. A PCR-based methodology for genotyping *bpk* mice was also developed. No significant differences in KW, BW, KW/BW%, or BUN were seen in cystic mice with *versus* without TGF- α . Cystic kidney disease and liver disease histology were similar. AR, EGF, HB-EGF, EGFR, and ErbB4 were abnormally expressed to an equal degree in kidneys of mice with *versus* without TGF- α . Although previous data suggest a critical role of TGF- α in murine PKD, these data show that TGF- α is not required for renal cyst formation or kidney or liver disease progression. We speculate that the therapeutic effect of WTACE2 could have been due to effects on several TACE targets, including TGF- α , AR, and ErbB4, as well as metalloproteinases other than TACE.

Abbreviations

ADPKD, autosomal dominant polycystic kidney disease; **AR**, amphiregulin; **ARPKD**, autosomal recessive polycystic kidney disease; **BUN**, blood urea nitrogen; **BW**, body weight; **EGF**, epidermal growth factor; **EGFR**, epidermal growth factor receptor; **HB-EGF**, heparin-binding epidermal growth factor; **KW**, kidney weight; **MMP**, matrix metalloproteinase; **PKD**, polycystic kidney disease; **TACE**, tumor necrosis factor-alpha converting enzyme; **TGF- α** , transforming growth factor-alpha

PKD is characterized by abnormal renal tubular epithelial cell proliferation, which contributes to cyst formation and expansion (1). Numerous studies have implicated the EGFR and its ligand, TGF- α , in the pathogenesis of this abnormal cell proliferation. EGFR and TGF- α are abnormally expressed in murine and human PKD (2–4). Transgenic mice that overexpress

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TGF- α develop cystic kidneys (5). TGF- α overexpression also accelerates the progression of cystic kidney disease in the *pcy* murine model of PKD (6). Treatment with novel inhibitors of EGFR tyrosine kinase significantly attenuated the severity of cystic kidney disease in the *bpk* mouse model of ARPKD and the Han-SPRD rat model of autosomal dominant PKD (ADPKD) (7,8). Treatment of cystic *bpk* mice with a novel inhibitor of TACE, the metalloproteinase that mediates TGF- α ectodomain shedding, significantly reduced the severity of cystic kidney disease (2). Furthermore, when compared with single higher dose EGFR inhibitor therapy, combination therapy with a TACE inhibitor and a decreased dose of an EGFR inhibitor was as effective (9), suggesting a synergistic effect of the two. Based on these collective findings, we hypothesized that TGF- α has an essential role in cyst formation and progression of cystic kidney disease in murine ARPKD.

In the current study, we sought to determine 1) whether TGF- α is an absolute requirement for renal cystogenesis in ARPKD and 2) whether the absence of TGF- α would diminish the severity of cystic kidney or biliary disease or modulate the expression of other EGF-related growth factors and their receptors. As a secondary aim, we sought to develop a PCR-based methodology for rapidly genotyping *bpk* mice, based on the recent identification of bicaudal C (*Bicc1*) as the mutated gene in this model (10).

METHODS

Animal models

The inbred mouse strain C57BL/6J-*Tgfa*^{tm1Ard}, which is homozygous for a TGF- α null allele (11), was obtained from the Jackson Laboratories (Bar Harbor, ME). TGF- α null (TGF -/-) mice develop normally and are fertile. Their phenotypic abnormality is characterized by pronounced waviness in the coat and whiskers, and dramatic derangement of the hair follicles (11,12).

The *bpk* model of murine ARPKD arose out of a spontaneous mutation in a Balb/C colony (13). Mice homozygous for the *bpk* mutation (*bpk* -/-) develop severe cystic renal disease evident histologically at birth and typically die by 28 d of age. The disease phenotype and course have been characterized previously (13,14). Mice heterozygous for the *bpk* mutation have no phenotypic abnormalities.

All animal experiments were conducted in accordance with policies of the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University. Reagents were obtained from Sigma Chemical (St. Louis, MO), unless otherwise indicated.

Breeding strategy and animal genotyping

Bpk heterozygote mice were bred with TGF- α null mice and the resultant offspring (all obligate TGF- α heterozygotes) were backcrossed to known *bpk* heterozygotes. Double heterozygotes (*bpk* +/-; TGF +/-) were then intercrossed to create cystic and noncystic offspring with 0, 1, or 2 TGF- α null alleles. Before the identification of the bicaudal C (*Bicc1*) as the *bpk* causative gene, and the development of a genotyping protocol (see below), *bpk* heterozygotes were identified based on a normal appearance and their ability to produce cystic offspring. Cystic (*bpk* -/-) mice were identified by the typical findings of massively enlarged, cystic kidneys corresponding to advanced cystic disease. When *Bicc1* was identified as the causative disease gene for the *bpk* mutation, a PCR-based genotyping protocol was developed (see below). Genomic DNA was isolated from tail samples and all offspring studied were then analyzed to confirm their *bpk* genotype. TGF- α heterozygote (TGF +/-) mice were identified by a normal appearance and PCR confirmation of the TGF- α null allele. The presence of the TGF- α null allele was identified by PCR for the *neo* cassette contained within the knockout construct, using

a standard genotyping protocol, "NEOTD," provided by the Jackson Laboratories and available at <http://jaxmice.jax.org>. TGF- α null mice were identified based on phenotypic changes (wavy hair, curly whiskers) and PCR confirmation of the TGF- α null allele. TGF- α wild-type (TGF +/+) mice were identified by a normal appearance and the absence of the TGF- α null allele.

Analysis of cystic kidney disease and liver disease severity

Kidneys were obtained at sacrifice from intercross progeny at d 21 of life, an age of advanced cystic collecting tubule disease in the *bpk* mouse, as described previously (13). Clinical parameters assessed included KW, BW, and KW/BW%. BUN was assessed by colorimetric assay using blood obtained by orbital puncture just before sacrifice. Data on clinical and laboratory parameters were presented as mean \pm SD and differences between groups were analyzed by two-tailed *t* test.

To assess histologic changes in the appearance of cystic kidney disease in mice with and without TGF- α , immunohistology was performed on 4- μ M sections obtained from paraformaldehyde-fixed plastic-embedded cystic kidneys, as described (with modifications) (7). Severity of cystic kidney disease was qualitatively assessed by staining serial sections with biotinylated lectins specific for collecting tubule (CT) [*Dolichos biflorus* agglutinin (DBA)] and proximal tubule (PT) [*Lotus tetragonolobus* (LTA)], and counterstained with hematoxylin, as described (2). The ratio of cystic CT to PT was also assessed, as described (2).

In our previous study involving treatment of cystic *bpk* mice with the TACE inhibitor, WTACE2, only the kidney disease severity was assessed (2). Therefore, the effect of TACE inhibition (*i.e.* TGF- α inhibition) on the liver phenotype was not known. However, treatment with a novel inhibitor of EGFR function has been shown to slow progression of the liver disease in this model (7). In the current study, we examined the impact of the absence of TGF- α on the liver disease in addition to the kidney disease. Livers were obtained at sacrifice and embedded as described above. Hematoxylin staining was performed on 4- μ M sections and hepatic (biliary) pathology was qualitatively assessed in cystic mice with and without TGF- α . Sections were examined for the presence of the characteristic bile duct ectasia and proliferation previously described in this model (13).

Expression of other EGF-related growth factors and their common receptor

Protein expression of the EGF-related growth factors, AR, EGF, and HB-EGF, and their receptors, EGFR and ErbB4, was examined by Western analysis. Total cellular protein was isolated from kidneys obtained at sacrifice, as described (2). Protein content of all samples was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). For AR and HB-EGF Western analysis, whole kidney lysates were subjected to heparin column purification, as described (15), before Western analysis. Samples were resolved by SDS-PAGE electrophoresis, transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA), and hybridized with blocking buffer (TBS/0.05% Tween 20/5% dry milk) followed by overnight incubation with rabbit polyclonal anti-human AR (Ab-1) (NeoMarkers, Fremont, CA) or rabbit polyclonal anti-rat HB-EGF (a gift from Dr. Raymond Harris, Vanderbilt University). Membranes were washed, hybridized with the appropriate peroxidase conjugated secondary antibodies, developed with ECL (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), and exposed to autoradiography film. For EGF, EGFR, and ErbB4 analysis, 100 μ g of total kidney lysate was diluted in SDS reducing buffer, resolved by SDS-PAGE electrophoresis, and immunoblotted, as above. Antibodies used were goat anti-mouse EGF (Santa Cruz Biochemicals, Santa Cruz, CA), sheep anti-EGFR (Upstate Biotechnology, Lake Placid, NY), and rabbit anti-ErbB4 (Santa Cruz Biochemicals).

Bpk genotyping protocol

To determine the genotype at the *bpk* locus, genomic DNA samples from tail clips were PCR-amplified using primers designed to flank the site of the *bpk* mutation on *Bicc1* exon 22, as outlined below. PCR reactions contained 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 130 μM each of *Bicc1* forward primer (5'-GGCATCTCTCCACT-GCTA-3') and reverse primer (5'-AGGTGCGTGTCCAGAAGGTA-3'), and 1U *Taq* Polymerase. PCR cycle parameters were 95°C × 3 min, 94°C × 2 min, 55°C × 2 min (30 cycles), 72°C × 1 min, 72°C × 10 min. Samples were cleaned using QIAquick PCR Kit (QIAGEN, Valencia, CA) and digested with BstU1 (New England Biolabs, Beverly, MA) for 2 h at 60°C. Restriction digest products were resolved on a 3% agarose gel stained with 0.5 μg/mL of ethidium bromide.

RESULTS

Absence of TGF-α does not prevent or reduce the severity of cystic kidney disease or liver disease

As shown in Figure 1, no differences were seen in the gross appearance of cystic or noncystic kidneys with TGF-α present compared with their TGF-α null counterparts. Table 1 summarizes the clinical parameters of each of the experimental groups. No significant differences in KW, BW, KW/BW%, or BUN values were seen in cystic mice with one or two copies of TGF-α compared with those without. Similarly, no changes in KW/BW% were seen in the noncystic mice with one or two copies of TGF-α *versus* those without. Noncystic TGF-null mice, however, did have lower KW (0.11 ± 0.02 *versus* 0.14 ± 0.02 , $p = 0.003$) and BW (6.87 ± 1.23 g *versus* 8.84 ± 0.88 gm, $p = 0.001$) compared with normal mice with TGF-α. Figure 2A demonstrates the histologic appearance of cystic kidneys with TGF-α (left panel) and without TGF-α (right panel). The severity of CT cysts, as shown by DBA staining, was similar in both cystic groups. A minority of cystic tubules in both cystic groups was PT in origin, as illustrated in the figure. The cystic CT to PT ratios were similar in mice with *versus* without TGF-α and the values were similar to those previously reported in kidneys from untreated cystic mice in our previous TACE inhibitor study (2). Figure 2B shows representative histology of livers from cystic mice with (left panel) and without (right panel) TGF-α, showing similar degrees of bile duct ectasia and proliferation.

Absence of TGF-α does not modulate the abnormal expression of other EGF-related growth factors and their receptors in cystic kidney disease

As noted previously, abnormal TGF-α and EGFR expression have been reported in both ARPKD and ADPKD. EGF expression abnormalities in PKD have also been described (16–20). In addition, we recently demonstrated abnormal AR and HB-EGF kidney expression in *bpk* mice (15). To determine whether the absence of TGF-α resulted in changes in the expression levels of other EGF-related growth factors or their receptors, Western analysis for AR, EGF, HB-EGF, EGFR, and ErbB4 was performed on whole kidney lysates obtained from cystic and noncystic mice with and without TGF-α. As shown in Figure 3, cystic mice (C) with TGF-α (TGF-α present) or without (TGF-α null) consistently showed similar abnormalities in the expression of all three growth factors as well as EGFR and ErbB4 when compared with their noncystic (N) counterparts. The abnormal expression of AR, HB-EGF, and EGFR is consistent with published data in ARPKD models (15,21). The EGF immunoreactive bands at approximately 18 and 28 kD differ in size from the more commonly reported protein species of 6, 47, and 170 kD (22,23); however, multiple different EGF species have been reported in PKD (16,17). Furthermore, the 18 and 28 kD species are overexpressed to an equal extent in cystic mice with *versus* without TGF-α and may represent an alternatively processed form of the protein. The ErbB4 abnormalities are consistent with our previous observations for ErbB4 (manuscript in preparation). Prominent expression of the full-length (170 kD) receptor, as well

as the constitutively cleaved (80 kD) protein species is present in cystic mice with and without TGF- α and only minimally visible in their noncystic counterparts.

Rapid genotyping of *bpk* litters

The *bpk* mutation has been previously identified in exon 22 of *Bicc1* (10), however, no published protocol exists for the rapid identification of this mutation from mouse genomic DNA. Using publicly available data from the Ensembl Mouse Genome Server (http://www.ensembl.org/Mus_musculus/) and from Genbank, genomic DNA sequences from the *bpk* locus and the *Bicc1* cDNA sequence (accession number NM_031397) were obtained and PCR was performed on DNA samples from known wild-type and homozygous mutant animals using primers flanking exon 22. Forward and reverse sequencing of the PCR products confirmed a CG insertion in *bpk* mutants, resulting in a frameshift mutation and loss of a stop codon in exon 22 as well as creation of a unique BstU1 restriction site (Fig. 4A). Primers were developed to flank the insertion/restriction site and genomic DNA samples from known wild-type, heterozygous, and homozygous mutant mice ($n = 3$ for each group) were amplified and cut with BstU1. As shown in Figure 4B, PCR amplification of the wild-type allele yields a 213 bp product, which is uncut by BstU1 digestion; whereas amplification of the mutant allele yields a 215 bp product, which is cut by BstU1 into 124 bp and 91 bp fragments.

DISCUSSION

To definitively test the hypothesis that TGF- α was an absolute requirement for renal cystogenesis and disease progression, we examined the impact of the loss of TGF- α expression on the development and severity of cystic kidney disease as well as biliary disease in the *bpk* model. Somewhat unexpectedly, the histologic and biochemical data of this study demonstrate that TGF- α is not required for renal cystogenesis in murine ARPKD and that the absence of TGF- α does not affect the severity of cystic kidney disease or biliary disease. One possible explanation for the lack of an effect on renal cyst formation in this model is that other EGF-related growth factors were up-regulated in response to the “loss” of TGF- α . We assessed kidney expression of EGF, AR, and HB-EGF as well as their receptors, EGFR and ErbB4, and did not find any significant differences in the expression of these growth factors and receptors in cystic mice with TGF- α versus those without. However, it should be noted that all of the growth factors and receptors studied were consistently abnormally expressed in cystic kidneys compared with noncystic kidneys, regardless of the presence or absence of TGF- α . This finding suggests that abnormal expression of one or more EGF-related growth factors is sufficient to induce cystogenesis and disease progression even in the absence of TGF- α .

These data further highlight the potential redundancy of the EGF-related growth factors/receptors in this disease. As noted previously, mice that overexpress TGF- α develop renal cysts (5). Our data suggest that up-regulation of one or more of the other EGF-related growth factors could have a similar effect. Similar findings were reported in studies examining the impact of single or combinatorial loss of AR, EGF, and TGF- α on mammary gland development (24). Despite the lack of all three growth factors studied, triple null mice were healthy and fertile. Furthermore, those lacking all three growth factors did not show significant changes in cellular proliferation within terminal buds. To our knowledge, transgenic mice that over-express AR and HB-EGF in the kidney have not been reported, however, transgenic mice that overexpress AR or HB-EGF in the pancreas demonstrate abnormal proliferation of ductal epithelium (25, 26). Similarly, mice that overexpress EGFR in the mammary glands develop mammary epithelial hyperplasia (27). In contrast, mice that widely overexpress EGF in multiple organs did not show any abnormalities (28), suggesting that not all of the EGF-related growth factors are able to initiate renal cystogenesis.

Modulation of disease by loss of TGF- α has been examined in several studies involving other organ systems. In a study of bleomycin-induced lung injury and fibrosis, TGF- α null mice showed a significant reduction in pulmonary fibrosis compared with mice with intact TGF- α (29). In that study, increased expression of HB-EGF was noted in both wild-type and TGF- α null mice treated with bleomycin compared with untreated controls but the protective effect of TGF- α loss was still seen. In a study of gastric ulceration in normal and TGF- α null mice, those without TGF- α failed to show the expected temporal increase trefoil peptide expression, suggesting that the latter stages of injury repair in this model had a TGF- α -dependent component (30). In contrast, in a study of peripheral nerve injury, TGF- α null mice had the same degree of response as those with intact TGF- α (31). These studies highlight the complexity of this growth factor/receptor axis and suggest that individual EGF-related growth factors may have specific and unique functions in certain tissues and cells, while showing redundancy of function in other organs.

In addition to causing renal cysts, TGF- α overexpression has also been implicated in worsening progression of disease in the *pcy* mouse, a purported model of human ADPKD (6). The fact that our data did not show any change in cystic phenotype would seem to be at odds with this previous report. However, it should be noted that although TGF- α overexpression accelerated disease progression in that model, neither the timing of initial cyst development nor the final degree of kidney enlargement were impacted by the presence of the TGF- α transgene (6). Of note, recent genetic studies have shown that the *pcy* mouse is actually a model of juvenile nephronophthisis (32), a cystic kidney disease of childhood that is genetically and phenotypically distinct from ARPKD. Furthermore, the fact that the overexpression of TGF- α accelerated disease in that model, does not mean that its absence would retard disease, given the redundancy of this growth factor system as discussed above. The timing of overexpression during the course of development may also impact the effect of growth factor on disease progression. In the *bpk* mouse, administration of exogenous EGF before 9 d of age improved renal function but not cystic kidney appearance, whereas, when treatment was continued beyond 9 d, mortality actually increased in treated *versus* untreated cystic mice (33).

The fact that TGF- α is not an absolute requirement for renal cystogenesis and that multiple EGF-related growth factors are abnormally expressed in murine ARPKD raises the question of how the previously studied TACE inhibitor, WTACE2, exerted its therapeutic effects in this model (2). As mentioned above, TACE is a metalloproteinase that mediates ectodomain shedding of TGF- α (34). More recent data have shown that other members of the EGF-related growth factor “family” also undergo metalloproteinase-mediated proteolytic cleavage to release the mature “secreted” growth factor [reviewed in (35) and (36)]. One possible reason for the observed therapeutic efficacy of WTACE2 was its actions on the shedding of other growth factors. Published data demonstrate that AR is a target of TACE (37). More recent studies demonstrate that HB-EGF and ErbB4 (which is unique among the ErbB receptors in that it undergoes proteolytic cleavage) are also targets of TACE (38,39).

Although WTACE2 is a potent inhibitor of TACE, it is not completely specific for TACE. Other metalloproteinases that are targets of the WTACE2 include MMP-3 and MMP-9 (2), both of which are overexpressed in PKD (40). Thus, the therapeutic effects of WTACE2 could have been due, in part, to inhibition of several other metalloproteinases in addition to TACE.

The *bpk* mouse is a well-characterized model of murine ARPKD and has consistent phenotype. These investigations highlight its usefulness in studying the pathogenesis of ARPKD. The PCR/restriction digest protocol developed in this study allows for rapid genotyping of whole animals, tissues, and cells. This will facilitate breeding experiments, future studies of therapeutic interventions, and studies involving cell lines derived from this model (41).

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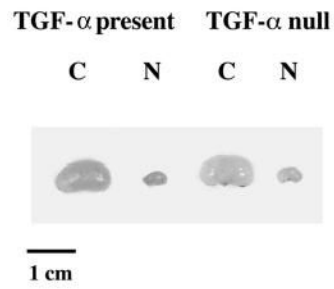


Figure 1.

Whole kidney micrographs from d-21 cystic (C) *bpk* mice and noncystic (N) littermates with TGF- α present or absent. Micrograph demonstrating relative kidney sizes of cystic *bpk* mice and noncystic mice with TGF- α (TGF- α present) or without (TGF- α null). Cystic kidneys with or without TGF- α are similar in size and markedly enlarged compared their noncystic counterparts.

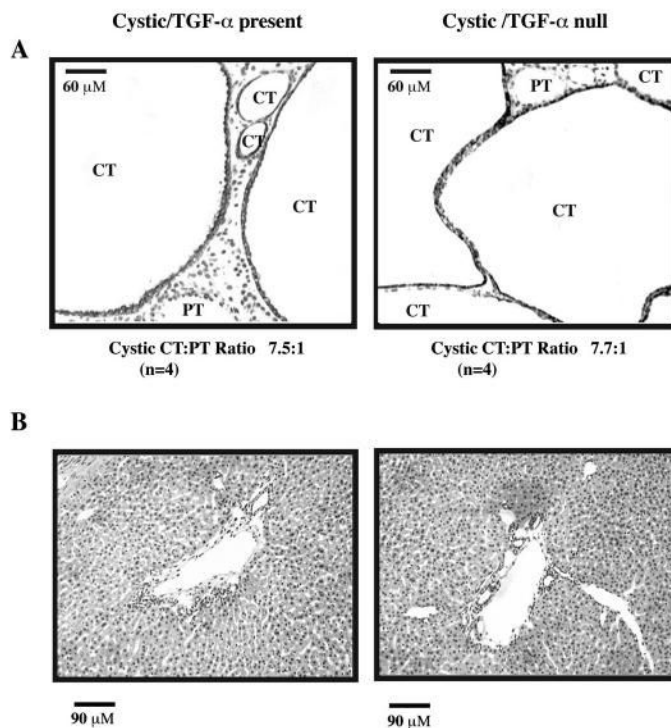


Figure 2.

Histologic appearance of d-21 kidneys and livers from cystic *bpk* mice with TGF- α present or absent. (A) Serial kidney sections from cystic mice with TGF- α (Cystic/TGF- α present) or without TGF- α (Cystic/TGF- α null) were stained with biotinylated *Dolichos biflorus* agglutinin (DBA), a CT marker or biotinylated *Lotus tetragonolobus* (LTA), a PT marker and counter-stained with hematoxylin as described in the text. Representative kidney histologies are shown. CT cysts (DBA+)/LTA- are indicated by dark staining and predominate in both groups studied. A minority of cystic PT (DBA-/LTA+) is present. The cystic CT:PT ratio is indicated below each panel. Neither the overall severity of cystic CT disease nor the appearance or proportion of cystic PT appears to be modulated by the presence or absence of TGF- α . CT, collecting tubule cyst; PT, proximal tubule cyst. Original magnification 20 \times . (B) Serial liver sections from cystic mice with TGF- α (Cystic/TGF- α present) or without TGF- α (Cystic/TGF- α null) were stained with hematoxylin as described in the text. Representative liver histologies are shown and demonstrate that the severity of bile duct ectasia and proliferation was qualitatively similar in mice with versus without TGF- α . Original magnification 20 \times .

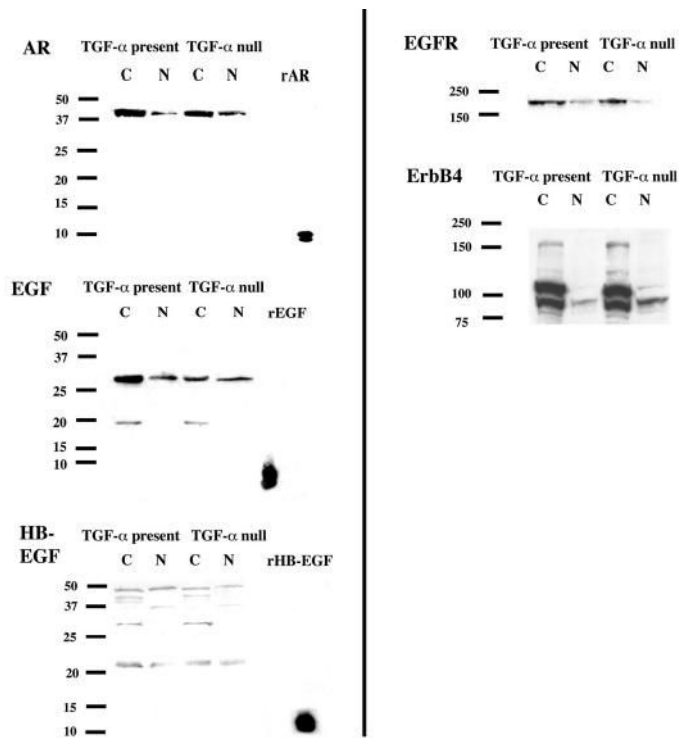
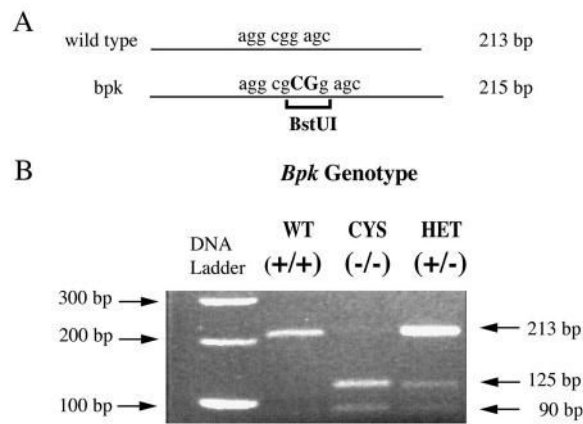


Figure 3.

AR, EGF, HB-EGF, EGFR, and ErbB4 expression in cystic *bpk* mice and noncystic littermates, TGF- α present or absent. Whole kidney lysates from d-21 cystic (*C*) and noncystic (*N*) mice with TGF- α (TGF- α present) or without (TGF- α null) were prepared as described in the text. Western analysis for AR, EGF, HB-EGF, EGFR, and ErbB4 protein expression was performed, also as described. AR and HB-EGF whole kidney lysates were subjected to heparin column purification before loading. Recombinant purified growth factor was loaded as a positive control for EGF-related growth factor immunoblots. Expression of AR, EGF, HB-EGF, EGFR, and ErbB4 is increased to similar degrees in cystic mice with or without TGF- α when compared with their noncystic counterparts.

**Figure 4.**

Rapid genotype analysis of *bpk*. (A) Schematic representation of *Bicc1* exon 22 showing the location of the *bpk* mutation (CG insertion) and creation of a unique *Bst*UI restriction site. (B) PCR amplification with primers flanking the mutation site, followed by *Bst*UI restriction digest of PCR products clearly distinguishes between wild-type, homozygous, and heterozygous DNA samples. The wild-type allele yields a 213 bp product, which is not cut by *Bst*UI. The *bpk* mutant allele yields a 215 bp product, which is cut into 125 bp and 90 bp fragments. *WT*, wild type; *CYS*, cystic; *HET*, heterozygote.

Table 1

Clinical parameters of experimental groups

Phenotype/genotype	No.	KW (g)	BW (g)	KW/BW (%)	BUN (mg/dL)
(A) Cystic/TGF- α present					
bpk $^{-/-}$ TGF $^{+/-}$	22	2.23 \pm 0.64	8.92 \pm 1.18	24.8 \pm 5.1	93 \pm 30
bpk $^{-/-}$ TGF $^{+/+}$	18	2.22 \pm 0.67	8.90 \pm 1.26	24.8 \pm 5.1	89 \pm 19
(B) Noncystic/TGF- α present					
bpk $^{-/-}$ TGF $^{+/+}$	4	2.26 \pm 0.54	9.13 \pm 0.87	24.8 \pm 6.0	108 \pm 52
bpk $^{+/-}$ TGF $^{+/-}$	48	0.14 \pm 0.02	8.84 \pm 0.88	1.60 \pm 0.13	22 \pm 4
bpk $^{+/-}$ TGF $^{+/+}$	25	0.14 \pm 0.02	8.71 \pm 0.74	1.57 \pm 0.13	23 \pm 5
bpk $^{+/+}$ TGF $^{+/-}$	11	0.15 \pm 0.02	9.29 \pm 1.00	1.62 \pm 0.14	22 \pm 4
bpk $^{+/+}$ TGF $^{+/+}$	8	0.14 \pm 0.02	8.74 \pm 1.08	1.61 \pm 0.11	20 \pm 4
(C) Cystic/TGF- α null					
bpk $^{-/-}$ TGF $^{+/-}$	4	0.14 \pm 0.02	8.62 \pm 0.86	1.64 \pm 0.17	22 \pm 6
bpk $^{-/-}$ TGF $^{-/-}$	11	2.18 \pm 0.51	8.5 \pm 1.0	25.6 \pm 5.2	106 \pm 21
(D) Noncystic/TGF- α null					
bpk $^{+/-}$ TGF $^{-/-}$	15	0.11 \pm 0.02 [*]	6.87 \pm 1.23 ^{**}	1.65 \pm 0.10	26 \pm 3
bpk $^{+/+}$ TGF $^{-/-}$	11	0.11 \pm 0.02 [*]	6.89 \pm 1.29 ^{**}	1.66 \pm 0.09	26 \pm 3
bpk $^{+/+}$ TGF $^{-/-}$	4	0.11 \pm 0.02 [*]	6.81 \pm 1.24 ^{**}	1.62 \pm 0.11	24 \pm 3

* $p = 0.003$, noncystic TGF absent vs present.** $p = 0.001$, noncystic TGF absent vs present.