Polycystic Kidney Disease as a Result of Loss of the Tuberous Sclerosis 2 Tumor Suppressor Gene During Development

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Somatic loss of function of the tuberous sclerosis 2 (TSC2) tumor suppressor gene leads to the development of benign and malignant lesions of the kidney, brain, uterus, spleen, and liver and germline loss of function of this tumor suppressor gene is embryonic lethal. In addition, the gene product of TSC2, tuberin, is necessary for normal function of the polycystic kidney disease 1 (PKD1) gene product, polycystin-1, which is required for normal cell-cell and cell-matrix interactions. We report here the development of severe polycystic kidney disease in three cases of young Eker rats carrying a germline inactivation of one allele of the *Tsc2* gene. Extrarenal tumors were also noted in the spleen and uterus of these animals, which was remarkable given their young age and in the case of the spleen, diffuse involvement of the affected organ. A cell line (EKT2) was established from an affected kidney of one of these animals and used in conjunction with tissues from affected animals to elucidate the defect responsible for the development of these lesions. Affected cells were determined to have lost the wild-type *Tsc2* allele while retaining two copies of chromosome 10 containing the mutant *Tsc2* allele along with two normal copies of the *Pkd1* gene. The genetic data, bilateral nature of the observed kidney disease, and extent of involvement of the spleen and kidney indicate that, in affected animals, loss of the wild-type *Tsc2* allele occurred during embryogenesis, probably as a result of chromosome nondisjunction, with affected animals being mosaics for loss of *Tsc2* gene function. (*Am J Pathol* 2003, 162:457–468)

The Eker rat has been extensively used as a uniquely susceptible animal model for renal cell carcinoma (RCC).^{1–6} These rats are susceptible to the development of spontaneous and carcinogen-induced RCC because

of a germline defect in the tuberous sclerosis 2 (*Tsc2*) tumor suppressor gene.^{3,5} In heterozygous Eker rats that inherit a mutant *Tsc2* allele (*Tsc2*^{EK/+}), RCCs develop after somatic inactivation of the normal *Tsc2* allele.^{7–13} These tumors typically appear between 10 and 12 months of age, with tumor incidence approaching 100% in *Tsc2*^{EK/+} carrier rats.^{2,12} In addition to developing RCCs, Eker rats also develop spontaneous tumors of the uterus (uterine leiomyoma), spleen (hemangiosarcoma), and pituitary gland.^{2,14–16} Less is known about the age of onset of the extrarenal tumors that develop in these animals, but tumors other than RCC in animals younger than 10 months of age have not been reported.

Homozygous deficiency of the *Tsc2* gene leads to embryonic lethality. *Tsc2*^{EK/EK} embryos die in midgestation, during the rat equivalent of mouse embryonic days 9.5 to 13.5, when *Tsc2* is strongly expressed in embryonic neuroepithelium.¹⁷ During this period, embryos lacking functional *Tsc2* display dysraphia and papillary overgrowth of the neuroepithelium, indicating that loss of *Tsc2* gene function disrupts the normal development of this tissue. This is also a key period for the development of the metanephric kidney, which emerges at approximately embryonic day 12 to 12.5. However, overlap between the window of lethality and this developmental window has precluded an examination of the effect of loss of the *Tsc2* gene on the development of the kidney.

Eker rat RCCs arise as a result of loss of function of the wild-type *Tsc2* allele, leading to the development of chromophilic lesions, primarily in the renal cortex. In spontaneous tumors, *Tsc2* inactivation generally occurs as a result of loss of heterozygosity (LOH) at the *Tsc2* locus,^{7,11,18} although point mutations in the carcinogen-induced RCC have also been reported.^{10,13,18} Loss of the normal *Tsc2* allele occurs early in the process of tumorigenesis¹⁹ and loss of the wild-type *Tsc2* allele in preneoplastic lesions coincides with overexpression of transforming growth factor (TGF)- α ,²⁰ which is also overexpressed in human RCC.^{21,22} Interestingly, renal cysts develop frequently in Eker rats,

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and so are a significant feature of tumor development in this animal model. Preneoplastic dysplasias, both spontaneous and carcinogen-induced, often develop as cystic lesions in both the proximal and distal nephron.²³ Carcinogens such as the nephrotoxicant and nephrocarcinogen hydroquinine and its active metabolite Tris-hydroquinine (Tris-HQ) also induce renal adenomas and RCCs in Eker rats.¹⁹ Like spontaneous tumors, the tumors that develop in animals treated with these compounds have also lost the normal *Tsc2* allele.¹⁹ Laser capture microdissection has been used to show that LOH occurs in preneoplastic lesions induced with Tris-HQ.¹⁹ Preneoplastic lesions induced by Tris-HQ, dysplasias described as "toxic tubules," are primarily cystic. These lesions have proliferative polyploid projections of dysplastic cells from cyst walls of tubules that have undergone regeneration after injury.¹⁹ Both the adenomas and carcinomas that develop after loss of tuberin function also often have a prominent cystic component.²³ Thus, in the Eker rat model, there appears to be a link between cystagenesis and tumorigenesis, implicating the *Tsc2* gene in both processes.

Recently, we identified a functional link between tuberin and polycystin-1, the product of the polycystic kidney disease 1 (*Pkd1*) gene.²⁴ Autosomal-dominant polycystic kidney disease is the most common potentially lethal genetic disease in humans.^{25–27} Most autosomal-dominant polycystic kidney disease occurs as a result of defects in the *PKD1* gene that result in loss of function of polycystin-1 and the development of epithelial cysts in the kidney, liver, and less frequently, other organs, including the pancreas, spleen, and arachnoid and seminal vesicles.^{28,29} The *TSC2* and *PKD1* genes are adjacent on human chromosome 16, and cases of extremely severe infantile autosomal-dominant polycystic kidney disease have been described in which the predisposing genetic alteration is a co-deletion of one allele each of *TSC2* and *PKD1*.^{30–32} In addition to this genetic evidence that these two genes participate in this disease, a functional linkage has been established between tuberin and polycystin-1. Tuberin is required for appropriate cellular localization of polycystin-1 to the basal-lateral aspect of the plasma membrane and in tuberin-null cells, polycystin-1 fails to localize to adherens junctions at the cell surface and becomes sequestered in the Golgi.²⁴ Loss of the *Tsc2* gene was shown to result in functional inactivation of polycystin-1 in the absence of genetic alterations at the *Pkd1* locus,²⁴ suggesting that in the Eker rat model, the cystic characteristic of preneoplastic lesions and tumors may be a result of functional inactivation of polycystin-1 in *Tsc2*-null cells.

In this report, we describe the consequence of loss of the *Tsc2* suppressor gene during development and the genetic basis for a severe form of polycystic kidney disease that occurs in very young Eker rats. In these animals, loss of tuberin as a consequence of LOH of *Tsc2* during embryogenesis resulted in a severe form of bilateral polycystic kidney disease and the development of extrarenal neoplasms in very young animals. We used *Tsc2* and *Pkd1* polymorphisms to assess the genetic status of both of these genes in kidney lesions from affected animals and EKT2, a cell line derived from poly-

cystic kidney epithelial cells. These data established that while the *Pkd1* gene was intact in affected animals, LOH at the *Tsc2* locus resulting from chromosome duplication and nondisjunction during embryonic development resulted in mosaicism for loss of tuberin function, subsequent development of polycystic kidney disease, and neoplastic transformation of renal tubular epithelial cells.

Materials and Methods

Cell Lines and Culture

The EKT2 cell line was established from a polycystic/neoplastic kidney of Eker rat CIIT-2. EKT2 cells were grown in DF8/3T3-conditioned medium³³ containing 10% fetal bovine serum and were maintained at 37°C in a humidified incubator with 5% CO₂. To determine the doubling time for EKT2 and the ERC18 cell line,¹² cells were plated in triplicates in 24-well dishes at 5×10^4 per well in growth medium. Cells were harvested by trypsin at 24-hour intervals and counted with a Coulter counter (Coulter Corp., Hialeah, FL). Doubling times ($t_{1/2}$) were calculated from the logarithmic portion of the growth curves.

Karyotyping and Fluorescence *in Situ* Hybridization

Karyotyping and fluorescence *in situ* hybridization analysis were performed to confirm the status of *Pkd1* alleles in EKT2 cells. Cells were disassociated by trypsin treatment, centrifuged at $300 \times g$ for 5 minutes, resuspended in 7.5 mmol/L of KCl at room temperature for 15 minutes, and fixed in methanol:glacial acid (3:1, v:v) mixture. Cells were washed three times with fresh fixative, placed on microscope slides and air-dried. Metaphase chromosomes were analyzed by quinacrine/Hoechst 33258 staining. The PKD1 probe for fluorescence *in situ* hybridization analysis was labeled with biotin-16-dUTP (Boehringer Mannheim, Indianapolis, IN) by nick-translation, purified by ethanol precipitation, and dissolved in 20 μ l of formamide. The probe was denatured at 95°C and mixed with hybridization buffer consisting of one part of bovine serum albumin (Boehringer Mannheim), two parts of 10 $\times$ standard saline citrate (SSC), and two parts of 50% dextran sulfate (Sigma, St. Louis, MO). Denatured chromosomes were incubated with hybridization buffer containing labeled probe at 37°C for 15 hours in a humidified chamber. After hybridization, the slides were washed sequentially at 37°C in 50% formamide/2 $\times$ SSC, 2 $\times$ SSC, and 1 $\times$ SSC for 15 minutes, with a final wash in 4 $\times$ SSC for 5 minutes. The slides were immersed in 70 μ l of 4 $\times$ SSC containing 3 μ g/ml of fluorescein isothiocyanate-avidin (Vector Laboratories, Burlingame, CA), and 1% bovine serum albumin for 45 minutes at 37°C. The slides were washed for 5 minutes in 4 $\times$ SSC, 4 $\times$ SSC containing 0.05% Triton X-100, and 4 $\times$ SSC, mounted in anti-fade solution [10% phosphate-buffered saline (PBS), 1% diazabicyclooctane, 1 μ g/ml 4',6'-diamidino-2-phenylindole, and 1 mg/ml *p*-phenylenediamine, all from Sigma].

Labeled chromosomes were analyzed with an Olympus fluorescence microscope.

Tumorigenicity

Tumorigenicity of EKT2 cells was assessed using the nude mice assay. Mice were injected subcutaneously at two sites per animal with EKT2 cells suspended in 0.5 ml of sterile PBS. Six mice were injected with 10^6 cells and another six received 0.5×10^6 cells per site. After 3 months, all of the mice were terminated using CO₂ euthanasia and examined for lesions. In addition to visual examination, injection sites were dissected and evaluated histologically.

Western Blot Analysis

For tuberin detection, tissues and cells were lysed in lysis buffer [PBS containing 0.1% sodium dodecyl sulfate, 1% Nonidet P-40, 0.5% deoxycholic acid, and 1 mmol/L phenylmethanesulfonyl fluoride (Sigma), 1 μ g/ml aprotinin (Sigma), 1 μ g/ml leupeptin (Sigma)] on ice for 60 minutes and centrifuged at $10,000 \times g$ at 4°C for 20 minutes to remove the nuclei. Protein concentration was determined with BCA Protein Assay Reagent (Pierce, Rockford, IL). Thirty μ g of protein from each lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred to polyvinylidene difluoride membranes. Nonspecific antigens were blocked by incubation at room temperature for 1 hour with 5% nonfat milk in TBST buffer (20 mmol/L Tris-HCl, pH 7.4, 136 mmol/L NaCl, and 0.05% Tween-20). The membranes were incubated with tuberin antibody C-20 (diluted 1:250; Santa Cruz Biotechnology, Santa Cruz, CA) for 2 hours in 1 $\times$ TBST containing 0.5% bovine serum albumin, washed three times in TBST at room temperature for 10 minutes and once in TBS, and incubated with secondary horseradish peroxidase-conjugated anti-rabbit antibody (diluted 1:2000; Santa Cruz Biotechnology) for 1 hour in 1 $\times$ TBST containing 1% nonfat milk. The membranes were washed three times in 1 $\times$ TBST, and tuberin was detected by using the LumiGLO Chemiluminescence Substrate Kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

RNA and DNA Isolation

Affected tissues were dissected free of normal tissue, frozen in liquid nitrogen, and ground with a mortar and pestle under liquid nitrogen. Cultured cells were washed twice with phosphate-buffered saline (PBS), then removed directly from their plates by scraping into guanidine isothiocyanate according to standard methods. Total RNA and genomic DNA were extracted from the cell lines and frozen tissues with the RNeasy and DNeasy kits (Qiagen Inc., Valencia, CA) and quantitated spectrophotometrically. Genomic DNA was also isolated with the DNeasy Kit (Qiagen Inc.) from formalin-fixed and paraffin-embedded tissues that were deparaffinized with xylene.

Northern Analysis

Aliquots of poly(A)⁺ RNA (5 μ g) from EKT2 cells and normal rat kidney were denatured, separated in 1.0% formaldehyde-agarose gels, transferred to nitrocellulose membranes by capillary blotting, and baked at 80°C for 2 hours, according to standard methods. cDNA probes for rat TGF- α ³⁴ and GAPDH were labeled with [α -³²P]dCTP to a specific activity of $>10^8$ cpm/ μ g by random oligonucleotide priming according to the manufacturer's recommendations (Prime-It II; Stratagene, La Jolla, CA). Hybridization with nitrocellulose membranes was performed in 40% formamide/3 $\times$ SSC at 42°C. Washes were at 35°C in 1 $\times$ SSC/0.1% sodium dodecyl sulfate. Membrane filters were exposed to X-ray film with intensifying screens for 48 to 72 hours.

Southern Blot and DNA Genotyping

High-molecular weight genomic DNA was isolated from tissues and the EKT2 and ERC15 cell lines by proteinase K (Promega, Madison, WI) digestion at 37°C for 16 to 18 hours, extraction with phenol/chloroform/isoamyl alcohol (24:24:1, v:v:v), and treatment with DNase-free RNase (Boehringer Mannheim). The DNA was ethanol precipitated, dissolved in TE buffer (pH 8.0), and quantitated spectrophotometrically. Fifteen μ g of each DNA sample was digested with XbaI and BglII, electrophoresed in 1.0% agarose gels, and transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Hybridization was performed in 5 $\times$ SSC containing 1 $\times$ Denhardt's, 50 mmol/L KPO₄, 0.25% (W:V) sodium dodecyl sulfate, 250 μ g/ml salmon sperm DNA (Ambion, Austin, TX), 50% formamide, and 10⁶ cpm/ml ³²P-labeled Pkd1 probe at 42°C overnight. The membranes were washed twice for 5 minutes each time in 1 $\times$ SSC at room temperature and four times for 30 minutes each time in 0.1 $\times$ SSC containing 0.05% sodium dodecyl sulfate at 50°C. The Pkd1 probes for Southern analysis were obtained by reverse transcriptase (RT)-polymerase chain reaction (PCR) using normal rat kidney RNA. RT-PCR products were gel-purified and labeled with [α -³²P]dCTP using the Prime-it II Random Primer Labeling kit (Stratagene). The primers used to generate the rat Pkd1 3' fragment were 5'-TGG ACA CCA CTC AGT ATT ACC-3' (forward) and 5'-CAC AGT AGT CCT GCC CTT GCT-3' (reverse). The primers used to generate the Pkd1 5' fragment were 5'-ACT TCC TCC CTG CCC ATT-3' (forward) and 5'-CCT GGT AAC CTT GGA GAC TA-3' (reverse). We examined EKT2 cells and tissues from Eker rats for Tsc2 LOH by using our previously developed PCR-high-performance liquid chromatography (HPLC) approach.¹⁸ Briefly, we performed PCR using a common forward primer for both the wild-type and mutant Tsc2 alleles (located in intron 30) and reverse primers specific for the mutant (located in the Eker mutation) and wild-type alleles (located in exon 31) for 35 cycles with denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, and elongation at 72°C for 30 seconds. Under these conditions, a 180-bp product specific for mutant Tsc2 allele and a

Table 1. Primers for Rat *Tsc2* cDNA Full Sequence

PCR fragment	Primer name	Primer sequence	PCR product (bp)	Exons	HPLC T _m (°C)
1	F	5'-CGGGAGCAGTTCTCTACAC-3'	428	E1-4	60
	R	5'-GAAGGATAGTCCTTGATCAC-3'			
2	F	5'-ACAGGGTGATCGTTTGGGGT-3'	401	E3/4-7	60
	R	5'-GGCACAGGGTGATAATGAAC-3'			
3	F	5'-GCTGTGGTCTGCTACAACCTGC-3'	388	E7-10	61
	R	5'-GCTCCTTCCTGTACTTCTTGA-3'			
4	F	5'-GTGGTGTGATGAGATTGTTTC-3'	345	E10-12	62
	R	5'-CAACTGCAGGTTCTGGATCCAGC-3'			
5	F	5'-GATAACCTACAGAGCCAGT-3'	382	E12-15	62
	R	5'-GTACACAGCCAGATCCCTTT-3'			
6	F	5'-GGACATCATTGAAAAGGTGA-3'	419	E14/15-18	63
	R	5'-CAGTTGGCGGTGAAAGGGGT-3'			
7	F	5'-TGAAGTGCAGGAACTGGACA-3'	394	E17/18-20	64
	R	5'-GTGGTAAGATATTAACGCTG-3'			
8	F	5'-GCTTCTCCAGAACTGACTTGC-3'	431	E20-23	60
	R	5'-CCTAATGAACCACATGGCTA-3'			
9	F	5'-CTCCAAGTTCAACCAGTACAT-3'	392	E22/23-26	61
	R	5'-CCATAGAGTTCTCATCCGCA-3'			
10	F	5'-GCAGAATACAGACATCTCTT-3'	380	E25/26-28	62
	R	5'-CTGGGACTCCAGTTTGGCCG-3'			
11	F	5'-GCTCAGCATCAAGCTCTGAT-3'	409	E27/28-30	64
	R	5'-GGCATGTTGTTGATGTCTGA-3'			
12	F	5'-CAGCTGGCTGATGAGCTTGA-3'	401	E30-32/33	63
	R	5'-AAGACCTGCTGTAGGCGTCAG-3'			
13	F	5'-CTTTGAGGCAGCATTAGGTA-3'	213	E32-33	64
	R	5'-GAAGAGCTAGACTTGCTCAAAGGC-3'			
14	F	5'-CTTTGAGCAAGTCTAGCTCTT-3'	385	E33-34	61
	R	5'-GAATGGTAGAGCTGTAGGAAC-3'			
15	F	5'-CAGTGCTGAAAAGGTGCCAGG-3'	399	E33-36/37	61
	R	5'-GCAATGTGGAAAACAGCTT-3'			
16	F	5'-GATGGACAGTTCACGTACTGC-3'	362	E36-39/40	61
	R	5'-GAGGCCATATTTGCATGCAGG-3'			
17	F	5'-CAGTGTGGCCAAGATTGTGT-3'	405	E39-41	63
	R	5'-CAGTCAGGAGGTGTCATTCAG-3'			

240-bp product specific for the wild-type *Tsc2* allele were amplified. After amplification, 5 to 10 μ l of PCR mixture were analyzed on the WAVE HPLC system with a DNA separation column (Transgenomic, Santa Clara, CA) under nondenaturing conditions. Under these conditions, PCR fragments were separated by HPLC based on their size, with the 180-bp product being eluted at 4.3 minutes and the 240-bp product eluted at 5.3 minutes.

Tsc2 Polymorphism Detection by Denaturing HPLC (DHPLC)

To identify DNA polymorphisms in the wild-type and mutant *Tsc2* alleles, we amplified 17 overlapping RT-PCR fragments covering the entire coding region of the *Tsc2* gene by using the primer pairs shown in Table 1. The RT-PCR was performed according to standard protocols and the annealing temperatures for PCR were calculated for each pair of primers based on their sequence. To detect the heteroduplexes, the RT-PCR products were denatured at 95°C for 5 minutes and allowed to reanneal by slow cooling to room temperature. Five to 10 μ l of each RT-PCR mixture was analyzed by denaturing HPLC (DHPLC). The gradient of acetonitrile and melting temperature (Table 1) for each RT-PCR *Tsc2* fragment were determined with WAVE 4 software (Transgenomic) de-

pending on the fragment sequence and size. Three alternative melting temperatures (predicted, 1°C lower, and 1°C higher than predicted) were used to confirm the presence or absence of heteroduplexes indicative of a nucleotide difference. To identify the specific nucleotides that differed between the wild-type and mutant *Tsc2* alleles, we sequenced the fragments that formed heteroduplexes with an ABI 377 automatic sequencer by using standard methods.

Results

Lesions in Eker Rats

We observed three instances of animals from colonies of Eker rats (CIIT-1, CIIT-2, UTMDA-3), two males and one female, that became moribund or died at an extremely young age (< 3 months) with severe bilateral renomegaly. Necropsy of these animals revealed extensive bilateral polycystic kidney disease. The characteristic macroscopic finding was severe bilateral renomegaly with cyst formation. In each animal, both kidneys were massively enlarged and exhibited diffuse bilateral involvement, with the exception of UTMDA-3, which had bilateral disease but segmental involvement of the right kidney. The affected portions of the right and left kidneys

of UTMDA-3 were identical in macroscopic appearance to the diffuse bilateral renomegaly in CIIT-1 and CIIT-2. The kidneys were enlarged with multiple cysts that were generally spherical and unilocular and ranged from 1 mm to 1 cm in diameter. A few cysts were multilocular and cylindrical or fusiform. The kidneys maintained a reniform shape and had a smooth cortical surface. On cut surface, the cysts were distributed homogeneously throughout the cortical and medullary regions and obliterated the normal cortical and medullary zones. Intercystic tissue was abnormal in color and consistency and appeared as gray firm infiltrate. Gray nodular foci (1 mm to 1 cm in size) were scattered throughout the cortical and medullary regions in intercystic regions. The renal pelvis and papilla were not discernable structures with the exception of the unaffected region of right kidney of UTMDA-3.

Microscopic examination of the massively enlarged kidneys revealed a polycystic renal parenchyma with relatively little normal kidney tissue (Figure 1). The most prominent feature of the admixture of cysts was that the epithelial lining of the cyst walls consisted of proliferative neoplastic cells. Small cysts were lined by so-called "chromatophilic renal tubular epithelial cells" that piled up in multiple layers to form "atypical hyperplasias." These dysplasias have been described previously as "preneoplastic" lesions in the rat.²³ In some instances, these lesions consisted of a simple layer of cuboidal to low-columnar epithelium, whereas in other areas the lesions formed intraluminal papillary excrescences that obliterated the cystic spaces. The larger affected regions were similar to cystpapillary renal cell adenomas and, in some areas, carcinomas because of the central areas of necrosis and cellular atypia. Throughout the kidneys the normal renal parenchyma was replaced by cysts whose walls contained an admixture of histological tumor patterns including solid, tubular, cystpapillary, trabecular, and comedo variants. There were no discrete areas of neoplasia within affected kidneys that exhibited instead a diffuse involvement. Normal renal structures including glomeruli were limited to intercystic cortical and medullary regions, except in the unaffected region of the right kidney of UTMDA-3, which had relatively normal renal parenchyma at the ventral pole.

The histological pattern and cytological appearance of the neoplastic cells were identical to those noted in Eker rats that develop solitary and multifocal spontaneous and carcinogen-induced neoplasias. These chromatophilic neoplastic renal cells were identical to the basophilic and eosinophilic subtypes previously described in this rodent model.^{2,20} However, in addition to the severe polycystic disease, the maintenance of a reniform shape and

Figure 1. A: Cross-section of cystic kidney showing smooth capsular surface and obliteration of normal renal architecture by round to cylindrical cysts extending throughout the cortical and medullary regions. **B:** Low-magnification photomicrograph demonstrates cysts lined by simple dysplastic epithelium as well as cysts with mural thickening caused by layers of piled up neoplastic epithelial cells. Solid areas of renal neoplasia forms the intercystic tissue. **C:** High magnification of neoplastic tissue showing admixture of basophilic and eosinophilic cytological subtypes of chromatophilic renal cell tumors. H & E; original magnifications: $\times 3.5$ (A); $\times 60$ (B); $\times 240$ (C).

smooth capsular surface of the polycystic kidneys was unique to these affected animals.

In UTMDA-3, additional tumors were observed in both the uterus and spleen, which are very frequent sites of tumor development in older *Tsc2*^{Ek/+} rats but have not been previously reported to develop tumors in such young animals. The histological appearance of these proliferative lesions in young Eker rats was similar to that previously reported in older *Tsc2* mutant rats. Interestingly, however, the angiomatous proliferation in the spleen of UTMDA-3 presented as a diffuse organ involvement rather than as a focal circumscribed lesion previously noted in the adult-onset cases. Microscopically the splenic lesion was characterized by diffuse proliferation in red-pulp regions of vascular channels lined by endothelial cells devoid of cellular atypia (Figure 2). Histologically, these splenic vascular proliferations have been described as benign vascular neoplasms (hemangiomas) although they may represent a hamartomatous change.² The extensive bilateral polycystic kidney disease in each of the affected animals, the diffuse involvement of the spleen with vascular proliferation, and involvement of multiple organs in such young animals, suggested that a genetic alteration during embryogenesis, rather than multiple independent somatic events, was responsible for the observed pathophysiology.

Characterization of EKT2 Cells

We established a primary explant from a portion of the polycystic kidney from animal CIIT-2 to facilitate the phenotypic and genotypic analysis of affected cells.²⁴ To characterize this cell line, cells from this explant were cultured and serially passaged *in vitro* >100 population doublings to derive the cell line designated EKT2. We determined the karyotype, doubling time *in vitro* and tumorigenicity *in vivo* to characterize the phenotype of EKT2 cells, and determined how they compared to RCC-derived cells in which *Tsc2* was inactivated somatically.

As shown in Figure 3, EKT2 cells were nearly diploid, except for a single translocation involving chromosomes 4 and 6 [+der(6)t(4:6)(q11:q24)] resulting in trisomy of the q arm of chromosome 4. EKT2 cells also contained two cytogenetically normal copies of chromosome 10, where both the *Tsc2* and *Pkd1* genes are located. Using a cosmid containing the rat *Pkd1* gene, we were able to demonstrate by fluorescence *in situ* hybridization analysis that EKT2 cells retained two copies of the *Pkd1* gene (data not shown). Consistent with their near diploid karyotype, EKT2 cells were not tumorigenic in nude mice (0 to 24 sites injected), similar to RCC-derived cell lines from Eker rats.¹² These cells had a doubling time of 26 hours, and similar to other cell lines exhibiting LOH at the *Tsc2* locus, EKT2 cells also overexpressed TGF- α (Figure 4),

Figure 2. **A:** Cross-section of enlarged spleen depicting expansion of red pulp by large blood-filled spaces. **B:** Low-magnification photomicrograph through affected splenic tissue showing proliferation of spindle-shaped mesenchymal cells interspersed between blood-filled vascular channels. **C:** High-magnification photomicrograph depicting capillary and cavernous channels lined by plump ovoid endothelial cells with occasional hyperchromatic nuclei. H & E; original magnifications: $\times 10$ (**A**); $\times 60$ (**B**); $\times 240$ (**C**).

Figure 3. Karyotype of the EKT2 cell line. A q arm trisomy of chromosome 4 resulted from a single translocation involving chromosomes 4 and 6 [+der(6)t(4:6)(q11:q24)] (shown in the **rectangle**). Other chromosomes, including chromosome 10 where the *Tsc2* and *Pkd1* genes reside, are diploid.

confirming that expression of this growth factor is associated with loss of *Tsc2* gene function.^{20,35}

Lack of Tuberin Expression in Affected Lesions

To directly assess the tuberlin status of the EKT2 cell line and affected and unaffected tissues, Western analysis was performed on frozen tissues and cell lysates. As previously reported for EKT2 cells, the affected regions from both the right and left kidneys of UTM DA-3 and the uterine leiomyoma from this animal did not express tuberlin, whereas several unaffected tissues, including the brain and liver did (Figure 5). Because EKT2 cells retained two copies of chromosome 10, lack of tuberlin expression was not because of monosomy 10. Therefore, we performed a genetic analysis to determine the mechanism responsible for loss of tuberlin in the affected lesions and EKT2 cells.

Tsc2 and *Pkd1* Polymorphisms

Originally the Eker mutation was identified in the Wistar strain of rats and was subsequently crossed and main-

Figure 4. Northern analysis of TGF- α . TGF- α is overexpressed in EKT2 cells compared with normal kidney of Eker rat.

tained on a Long-Evans background. We hypothesized that polymorphisms in the *Pkd1* gene that we had previously identified²⁴ were because of the retention of sequences characteristic of its Wistar origin. Therefore, while the wild-type *Tsc2* allele was from the Long-Evans background, we analyzed the full coding region of the *Tsc2* gene to identify strain-specific DNA polymorphisms that could discriminate between the Wistar and Long-Evans *Tsc2* alleles. Using 17 PCR primer pairs designed to span the entire coding region of the *Tsc2* gene (Table 1) and DHPLC, we identified a nucleotide change in fragment 4 (exons 10 to 12) (Figure 6). Sequencing of this fragment revealed that exon 10 of the wild-type *Tsc2* gene (the Long-Evans allele) contained a C at position 1092 (codon 364), whereas the inactive allele containing the Eker mutation (the Wistar allele) had a T at this position. The T-to-C substitution at position 1092 did not alter the amino acid sequence, indicating that this substitution is a DNA polymorphism between the Wistar and the Long-Evans rat strains.

We also characterized polymorphisms at the 3' and 5' ends of the *Pkd1* gene,²⁴ which lies 63 bases away from the *Tsc2* gene on rat chromosome 10,³⁶ as strain-specific polymorphisms (Figure 7). An *Xba*I polymorphism that discriminated between a 9.0-kb fragment in Long-Evans rats and a 5.6-kb fragment in Wistar rats at the 3' end of *Pkd1* and a *Bgl*II polymorphism that discriminated be-

Figure 5. Western analysis of tuberlin expression. A 180-kD tuberlin band is absent in EKT2 cells and in affected regions of both kidneys and uterine leiomyoma from UTM DA-3 animal. Normal renal epithelial TRKE2 cells, matching normal Eker rat tissues (control), and unaffected brain and liver from UTM DA-3 express tuberlin.

Figure 6. Rat *Tsc2* gene polymorphism analysis. Rat *Tsc2* cDNA was analyzed by DHPLC and direct sequencing. **A:** Schematic representation of *Tsc2* polymorphism in exon 10 (condon 364). **B:** DHPLC tracings showing a nucleotide difference between RT-PCR products (exons 10 to 12) amplified from *Tsc2*^{+/+}, *Tsc2*^{Ekl/+}, and *Tsc2*^{Ekl/-} cells. **C:** Corresponding partial sequence of *Tsc2* exon 10 from *Tsc2*^{+/+}, *Tsc2*^{Ekl/+}, and *Tsc2*^{Ekl/-} cells.

tween a 7.0-kb fragment in Long-Evans rats and a 7.4-kb fragment in Wistar rats at the 5' end of *Pkd1* were identified. That these polymorphisms could discriminate between *Pkd1* alleles adjacent to the mutant (Wistar) and the wild-type (Long-Evans) *Tsc2* alleles was demonstrated by using *Tsc2*^{Ekl/+} (ERC15 cells), which retain heterozygosity at the *Tsc2* locus and contain two *Pkd1* alleles (Figure 7).

Figure 7. Southern analysis of *Pkd1* polymorphisms between Long-Evans and Wistar rats. The black boxes indicate positions of the 3' and 5' *Pkd1* probes. The 3' probe recognizes 9.0- and 5.6-kb *XbaI* fragments in Long-Evans and Wistar *Pkd1* alleles, respectively (right blot). The 5' probe detects 7.4- and 7.0-kb *BglII* fragments in Long-Evans and Wistar *Pkd1* alleles, respectively (left blot). The gray box indicates position of the Eker mutation.

Loss of Wild-Type *Tsc2* Gene in Affected Cells

To determine whether inactivation of the wild-type allele of the *Tsc2* gene in the EKT2 cell line and affected kidneys occurred at the DNA level or the RNA level, we combined LOH analyses using a previously developed PCR-HPLC approach¹⁸ and the *Tsc2* exon 10 polymorphism described above. At the DNA level, the allele ratio for the 240-bp wild-type and 180-bp mutant PCR products from heterozygous cells was empirically determined by HPLC to be 1.23 ± 0.24 ($n = 24$) and LOH was defined as an allele ratio >1.71 (ie, >2 SD away from the mean).¹⁸ Calculation of allele ratio from the HPLC tracings shown in Table 2 demonstrated that both affected kidneys from UTMDA-3 and EKT2 cells had lost the wild-type *Tsc2* allele (Figure 8A). This finding was confirmed at the RNA level by using the *Tsc2* exon 10 polymorphism (Figure 8B). Both affected kidneys from UTMDA-3 and the EKT2 cell line expressed only RNA transcripts from the mutant *Tsc2* allele. Consistent with loss of the wild-type *Tsc2* allele, both EKT2 cells and affected kidneys exhibited loss of the *Pkd1* allele adjacent to the wild-type *Tsc2* gene (Figure 8C). However, Southern analysis demonstrated that EKT2 cells contained two copies of the Wistar *Pkd1* allele adjacent to the mutant *Tsc2* gene, consistent with the presence of two copies of chromosome 10 in these cells. Loss of the wild-type *Tsc2* allele in the polycystic kidney of CIIT2 from which EKT2 cells were derived was further confirmed by PCR analysis of paraffin sections from the affected kidney (Figure 9). The presence of two copies of chromosome 10 in EKT2 cells, both containing mutant *Tsc2* alleles, and the fact that the polycystic kidney disease in CIIT-2 was bilateral suggested that chromosome nondisjunction during embryogenesis caused the loss of the wild-type *Tsc2* allele. However, germline homozygosity for the Eker mutation resulting in loss of *Tsc2* function is embryonic lethal,¹⁷ suggesting that the affected animals were in fact mosaics.

To confirm the mosaicism of the affected animals, we performed DHPLC analysis for *Tsc2* LOH in affected and unaffected tissues from UTMDA-3 (Table 2). Similar to EKT2 cells and the affected kidney of CIIT-2, the affected kidney, spleen, and uterine leiomyoma from UTMDA-3 had lost the normal *Tsc2* allele and had only mutant *Tsc2* alleles (Table 2). Consistent with the absence of the wild-type allele, RNA isolated from affected kidney, spleen, and uterine leiomyoma was transcribed exclusively from the mutant allele, whereas RNA from unaffected tissues (stomach, brain, and liver) and the unaffected portion of the right kidney contained transcripts from both *Tsc2* alleles (Figures 8 and 10). These data indicate that affected animals were indeed mosaics, composed of affected organs containing cells that had lost the wild-type *Tsc2* allele (kidney, spleen, and possibly uterus) and normal organs (such as stomach, brain, and liver) that retained an apparently normal wild-type *Tsc2* allele. This mosaicism appears to be responsible for the development of severe, bilateral polycystic kidney disease and early onset lesions in the spleen and uterus in animals that were otherwise phenotypically normal.

Table 2. DHPLC Analysis of Affected and Unaffected Tissues

Tissues	Mut area	Wt area	Ratio (mut/wt)	Result
Brain	73685	68694	1.073	Heterozygous
Liver	65030	63223	1.029	Heterozygous
Stomach	70303	66733	1.053	Heterozygous
Small intestine	79397	75072	1.058	Heterozygous
Uterine leiomyoma	88325	38598	2.288	LOH
Neoplastic spleen*	43713	2598	16.826	LOH
Affected kidney	97695	31283	3.123	LOH
Unaffected kidney	77590	72818	1.066	Heterozygous

*Paraffin-embedded tissue.

Discussion

Here we report that very young Eker rats with a naturally occurring germline inactivation of the *Tsc2* tumor suppressor gene developed very severe polycystic kidney disease. Interestingly, except for the pronounced poly-

cystic nature of the lesion, the cytological and histological manifestation of the renal tubular neoplastic proliferation was identical to that noted normally seen in *Tsc2* adult-onset tumorigenesis. The neoplastic cells resembled the basophilic and eosinophilic chromophilic variants of renal cell tumors that have been previously described in this model.^{2,20} Massive organ involvement and coalescence of lesional area were observed. The presence of kidneys of a reniform shape with a smooth capsular outline made these cases quite different from the large RCCs that arise in adult Eker rats spontaneously or after carcinogen treatment. The multilocular cysts in these large juvenile lesions were also quite different from the cysts in most Eker rats, which develop cystadenomas and cystadenocarcinomas in adulthood.

Although the histological and cytological patterns of the neoplastic epithelial proliferations in the cystic kidneys in the cases described in this report were quite diverse, there were no other tumor types within the renal parenchyma. This is important because nephroblastomas, renal mesenchymal tumors, and transitional cell tumors have all been reported in rats and are histologically quite different from the epithelial tumors of the tubular epithelia. Nephroblastomas in rats have been well characterized and usually have a characteristic tripartite pattern, consisting of blastemal, epithelial, and stromal elements.³⁷ These are composed of nests, cords, and islands of poorly differentiated cells, morphologically resembling the embryonal renal blastema. In the cases reported here, despite early involvement of the kidney, there was no evidence of involvement of renal cell types other than differentiated renal tubular epithelial cells. None of the cystic kidneys had any evidence of nephro-

Figure 8. LOH analysis of the *Tsc2* and *Pkd1* genes in EKT2 cells and affected kidney of animal UTMDA-3. **A:** Quantitative PCR-DHPLC analysis showing LOH of the wild-type *Tsc2* allele in both the affected kidney and the EKT2 cell line. **B:** RT-PCR-DHPLC analysis of *Tsc2* exon 10 polymorphism demonstrates RNA expression from the mutant *Tsc2* allele. Direct sequencing confirmed that in both affected kidney and EKT2 cells, RNA is expressed from the mutant *Tsc2* allele. **C:** Southern analysis showed that the affected kidney and the EKT2 cell line have lost the *Pkd1* gene from Long-Evans allele (containing the wild-type *Tsc2* gene), while retaining the Wistar *Pkd1* alleles.

Figure 9. LOH analysis of the *Tsc2* gene in animal CIIT-2. PCR using DNA from paraffin-embedded section of the affected kidney of animal CIIT-2 from which the EKT2 cell line was derived amplified only a 180-bp product from mutant (mut) *Tsc2* allele, whereas both 240-bp and 180-bp products from the wild-type (wt) and mutant *Tsc2* alleles were detected in the paraffin-embedded normal Eker rat kidney.

Figure 10. *Tsc2* RNA expression in affected and unaffected tissues of UTMDA-3 animal. Using the *Tsc2* exon 10 polymorphism, expression of RNA from mutant *Tsc2* allele was detected by RT-PCR-DHPLC in uterine leiomyoma (A) and affected spleen (B). RNA transcripts from both wild-type and mutant alleles of the *Tsc2* gene were detected in unaffected kidney (C), brain (D), stomach (E), and liver (F).

blastoma development, nor any evidence of formation of other renal cell tumors such as the angiomyolipoma commonly noted in patients with tuberous sclerosis. The involvement of the spleen in UTMDA-3 was also unusual in that the lesion developed very early and had a diffuse red pulp involvement. The numerous well-formed vascular channels lined by relatively normal appearing endothelial cells made this lesion more reminiscent of a hamartomatous vascular proliferation rather than a true neoplastic

hemangioma. The role of *Tsc2* in vascular pathobiology remains to be determined, but vascular neoplasms or hamartomas are common in the spleen and occur more rarely in other visceral, subcutaneous, and dermal sites in Eker rats.^{2,38}

The bilateral nature of the polycystic kidney disease and extent of splenic involvement in the affected animals suggests that loss of the wild-type *Tsc2* gene occurred early in the development of these organs. Cytogenetic analysis and *Tsc2* and *Pkd1* LOH studies indicated that the affected cells contained two copies of the *Tsc2* gene carrying the Eker mutation and two copies of the adjacent *Pkd1* gene. The result was genetic inactivation of the *Tsc2* gene, loss of its gene product, tuberin, and apparent functional inactivation of polycystin-1, the product of the *Pkd1* gene that requires tuberin for normal function. The cytogenetic and LOH data suggested that chromosome nondisjunction may have been responsible for loss of the wild-type *Tsc2* gene in at least one of these cases, as the chromosome carrying the wild-type *Tsc2* gene was replaced with the one carrying the mutant allele. That a very young animal developed uterine leiomyoma, which normally develops in only mature females (>10 months old) is also consistent with an early loss-of-function event. In the kidney, the consequence of this apparent developmental loss of *Tsc2* was early development of severe renal cysts leading to polycystic kidney disease and neoplastic transformation of renal epithelial cells.

The cystic lesions in the polycystic kidney disease may be ascribed to loss of normal polycystin-1 function. As demonstrated previously, when polycystin-1 is expressed in the absence of tuberin, it becomes sequestered in the Golgi and fails to transit appropriately to the plasma membrane, resulting in functional inactivation of the protein.²⁴ Tubular dysmorphogenesis observed during the development of polycystic kidney disease has been hypothesized to result from altered extracellular matrix interactions of the tubular epithelial cells.³⁹ Polycystin is co-localized at the basolateral aspect of the cell membrane with E-cadherin and β -catenin in adherens junctions.^{40–42} Loss of polycystin localization to the plasma membrane in the absence of tuberin could potentially alter cell-cell and cell-matrix interactions, contributing to both the altered cell proliferation and altered production of extracellular matrix, which are hallmarks of polycystic kidney disease.

The *Pkd1* gene is 63 bp away from the *Tsc2* gene, and these two genes are transcribed in a tail-to-tail manner, with overlapping 3'-untranslated regions.⁴³ The *Tsc2* allele in which the Eker mutation occurs was originally derived from a Wistar rat.^{6,44} The spontaneous mutation that arose in these animals was subsequently crossed onto a Long-Evans background. We established that for the *Tsc2* exon 10 polymorphism, the mutant Eker allele is of Wistar origin and the wild-type allele is Long-Evans. Our data indicate that despite the large size of the *Tsc2* and *Pkd1* genes and fact that the Eker mutation has been retained on the Long-Evans background for >20 years, most likely these genes appear to be in linkage disequilibrium, as a result of their close proximity and the con-

tinued selection for the Eker mutation in this animal cancer model.

Most importantly, renal cells that had lost the *Tsc2* gene in the affected kidney were neoplastic cells of the type associated with the transformation of mature tubule epithelial cells (adenocarcinoma) rather than embryonic lesions (nephroblastoma) associated with transformation of developing metanephric kidney. Nephroblastoma is associated with loss of function of the Wilms' tumor suppressor gene (*Wt-1*).^{45,46} A previous study of Eker rats treated transplacentally with nitrosamines demonstrated that the induction of mutations in the *Tsc2* gene during kidney development very frequently results in early-onset RCCs that lack *Wt-1* mutations, rather than in nephroblastoma.⁴⁷ The proliferative renal tubular cyst linings with cytological and histological characteristics of transformed mature tubule epithelial cells in the polycystic kidneys reported here further suggests that the function of the *Tsc2* tumor suppressor gene is conditional on the stage of kidney development, affecting renal epithelial cells but not the nephroblasts from which they arise. These data suggest that loss of tuberlin function may have little consequence for nephroblasts until these cells have passed beyond the mesenchymal-epithelial transition and developed into epithelial cells. At this later period of kidney development, the absence of tuberlin has a dramatic impact, causing these epithelial cells to become transformed.

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