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Oval Cell Proliferation Associated with the Murine Insertional Mutation *TgN737Rpw*

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The* Tg737 *gene was identified by its direct association with a transgene-induced insertion mutation in the mouse. This mutation causes pleiotropic phenotypes including a syndrome similar to autosomal recessive polycystic kidney disease in humans. This syndrome, in addition to renal cyst formation, includes the presence of an invariably associated liver abnormality. The liver pathology in* TgN737Rpw *mice is characterized by a biliary hyperplasia that includes the proliferation of cells that morphologically and immunologically resemble oval cells, a liver progenitor cell. This abnormality is first observed at approximately 5 days of age in the portal region and then progresses into the periportal regions. Additionally, the formation and proliferation of dysplastic ductular structures are observed from the onset of the phenotype. Serum chemistry indicated that the primary defect is likely to be of biliary origin, and hepatic function appears normal in the mutant mice. Therefore, this mutation is unlike other causes of oval cell proliferation in that the hepatic parenchyma is relatively unaffected. The identification of the* Tg737 *gene associated with this mutation suggests that it functions in regulating the proliferation/differentiation of oval cells within the liver, which further indicates that this gene may function in pathological conditions that include oval cell proliferation, such as hepatocellular carcinogenesis. (Am J Pathol 1996, 149:1919–1930)

We previously described a transgene-induced insertional mutation in the mouse referred to as *TgN737Rpw*. This mutation disrupts the expression of the ubiquitously expressed *Tg737* gene that encodes a protein containing 10 copies of a 34-amino-acid tetratricopeptide-repeat (TPR) motif.[1,2] TPR motifs are structural moieties involved in mediating protein-protein interactions. They were first identified in yeast cell-cycle control proteins and since have been found in proteins with numerous functions including transcription, signal transduction and protein transport.[3] The disruption of the *Tg737* gene results in pleiotropic phenotypes that are transmitted in an autosomal recessive manner and are dependent on the genetic background of the animals. Interestingly, the salient feature of these phenotypes is the proliferation of epithelial cells. Among the traits exhibiting epithelial cell proliferation are polycystic kidneys and biliary hyperplasia in the mutant animals.

Biliary abnormalities have been described in a number of human conditions including autosomal recessive polycystic kidney disease (ARPKD)[4,5] which is a fatally inherited childhood disease defined by polycystic kidneys and a liver lesion characterized by a biliary dysplasia with an associated ductular and ductal hyperplasia and portal and perilobular

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fibrosis. The degree of hepatic fibrosis is variable between patients with ARPKD, and this heterogeneity is thought to reflect genetic background differences among ARPKD patients.

There are several murine polycystic kidney disease (PKD) models available and they have proved quite useful in studying the etiology of renal cyst formation.[6–10] However, relatively few of the murine ARPKD models exhibit an associated biliary abnormality. One that does is the BALB/c polycystic kidney (*bpk*) model.[11] Previously, we demonstrated that *TgN737Rpw* was not allelic to *bpk* nor does *Tg737* map to the chromosomal locations of other genes associated with mouse polycystic kidney models.[1] The major locus associated with human ARPKD has been localized to chromosome six,[12] whereas we demonstrated that the human homologue of *Tg737* (*hTg737*) maps to chromosome 13.[13] Furthermore, no heterogeneity in the population of individuals studied was observed when defining the map position of the ARPKD locus; therefore, it is unlikely that *Tg737* is the primary gene involved in ARPKD, but instead it may act in the same biochemical pathway. Nonetheless, the *TgN737Rpw* mouse provides an excellent model system to study biliary hyperplasia in general and that associated with renal cystic diseases in particular.

In rodents, the proliferation of nonparenchymal epithelial cells has been studied extensively through the use of hepatotoxic and hepatocarcinogenic regimens.[14] For example, the treatment of rats with the noncarcinogenic agent D-galactosamine causes the proliferation of a population of biliary epithelial cells that have characteristics of a less differentiated cell type referred to as oval cells.[15] Oval cells, so named because of their ovoid nucleus, have been studied extensively in rat models of liver carcinogenesis,[16–20] and an analogous cell type has been described in pathological conditions in human livers[21] as well as in mice.[22,23]

The role of oval cells in the normal liver as well as in pathological states has generated considerable controversy.[14,16,24,25] Whether oval cells represent a stem or progenitor cell population within the normal liver, which may be a target for liver carcinogens, is still unclear. Tumors derived from oval cells have been shown to contain cells with hepatocellular or biliary characteristics, suggesting that oval cells may be capable of differentiating down both cell lineage pathways.[14,20,26] This hypothesis has been tested further by using cultured oval cells to study the differentiation potential of this cell population both *in vitro* and *in vivo*. Results indicated that oval cells are capable of differentiating down both biliary and hepatocellular pathways,[27–32] suggesting that this cell type could function as a stem or progenitor cell in the liver. The observation that oval cells are detected only upon severe liver damage suggests that oval cells can act as a facultative stem cell population,[14] and others have proposed that oval cells may act in a stem cell lineage system within the liver.[16,33,34]

The identification of mutations that cause biliary hyperplasia and/or oval cell proliferation will provide valuable information into the development of various pathological conditions in the liver. The *TgN737Rpw* mutation causes biliary hyperplasia emanating from the portal triad. We present data indicating that the proliferating cells constitute a compartment of epithelial cells expressing different markers, some of which are expressed by oval cells. Furthermore, within the hyperplastic regions, a ductule proliferation occurs, suggesting that cells within these lesions are capable of differentiating down the biliary pathway. The proliferation of oval cells in the *TgN737Rpw* mouse in the absence of severe hepatic damage suggests that *Tg737* plays a direct role in regulating the proliferation/differentiation of this cell type.

Materials and Methods

Mice

The *TgN737Rpw* mouse model was generated as part of the large-scale insertional mutagenesis program at the Oak Ridge National Laboratory. Transgenic mice were generated on the FVB/N genetic background following standard pronuclear injection protocols.[35] All animals were handled in accordance with National Institutes of Health guidelines.

Histology

Livers from homozygous mutant, heterozygous, and wild-type *TgN737Rpw* mice were harvested at the indicated time points, fixed in neutral buffered formalin, and embedded in paraffin, and sections were prepared and subjected to hematoxylin and eosin (H&E) staining.

Immunohistology

The expression of α-fetoprotein (AFP) and cytokeratin (CK) was evaluated on formalin-fixed, paraffin-embedded liver samples as described elsewhere.[36] Primary antibodies were rabbit anti-keratin (A575) and anti-human AFP (N1501) polyclonal antibodies, both purchased from Dako Corp. (Carpinteria, CA). In addition, expression of a surface antigen common

to mouse biliary epithelial cells and oval cells was evaluated using a rat monoclonal antibody (A6) kindly provided by Dr. N. Engelhardt, Institute of Carcinogenesis, Cancer Research Center, Moscow, Russia.[22,37] All immunohistochemical evaluations were performed using the avidin-biotin peroxidase complex method (Vectastain PK-6100, Vector Laboratories, Burlingame, CA). Biotinylated antisera against rabbit or rat immunoglobulins (Vector Laboratories) were used as secondary antibodies. The reaction products were visualized with 3,3-diaminobenzidine (CK and A6) or 3-amino-9-ethylcarbazol (AFP) substrate kits, and Mayer's hematoxylin was used as a counterstain. Formalin-fixed, paraffin-embedded sections of mouse fetal liver and hepatocarcinomas were used as positive controls for AFP immunostaining.

Lectin Staining

Lectin staining using *Dolichos biflorus* agglutinin (DBA) and *Sophora japonica* agglutinin (SJA) were performed using biotinylated lectins (Dako) as probes and streptavidin-horseradish peroxidase to reveal the chromogen.

RNAse Protection Assay

Total RNA (10 μg) was subjected to RNAse protection using the RPAII kit from Ambion (Austin, TX) following the manufacturer's protocols. The probe consisted of a 400-bp fragment of *Tg737* and was transcribed using standard procedures. A hybridization temperature of 42°C was used.

Serum Chemistry

Blood was collected from metafane-anesthetized mice by retro-orbital bleeding. Serum was separated in Microtainer tubes (Becton Dickinson, Rutherford, NJ) following the manufacturer's instructions. The serum was stored frozen at −80°C until serum chemistry was performed at the University of Tennessee College of Veterinary Medicine using standard methods.

Electron Microscopy

Small pieces of liver were obtained from *TgN737Rpw* mice and littermate controls. The tissues were fixed overnight at 4°C in 2.5% glutaraldehyde, postfixed in 1% osmium tetroxide, and embedded in plastic. Thin sections were cut at 1 μm and strained with toluidine blue. Ultrathin sections were cut with an LKB ultratome, stained with uranyl acetate and lead citrate, and examined on a Phillips 300 electron microscope.

Results

Light Microscopy

The hallmark of the *TgN737Rpw* mutant liver phenotype on the FVB/N genetic background is the proliferation of a poorly differentiated epithelial cell population. The phenotype is completely penetrant but with variable expressivity. The time of appearance and severity of the phenotype may vary even within mutant mice born in the same litter. To determine when the epithelial cell phenotype first became apparent we isolated livers from mutant and control littermate mice from embryonic days 11.5 to 17.5 as well as postnatal time points. Sections of these livers were analyzed using light microscopy. No obvious differences between mutant and control livers were observed at any of the embryonic time points (data not shown). The first abnormalities observed in mutant livers were from 5-day-old neonatal mice (compare Figure 1a with 1b). The lesions were centered around the portal triads and were characterized by an excess of bile ductular structures and by the proliferation of nonparenchymal epithelial cells. By postnatal day 10 the proliferating cells expanded the portal triads (Figure 1c), and subsequently the proliferating cells expanded outward from the portal triad into the periportal area (Figure 1d, 16 days).

In mice that survived past weaning (55 of 497 mutant animals), the proliferating cells markedly expanded the portal region and delineated the hepatic lobules (Figure 2a). The proliferating epithelial cells can disrupt the limiting plate and invade into the hepatic parenchyma (Figure 2b). In the adult mutant liver, as in newborns, an excess of ductular structures was observed, especially in the portal region (Figure 2, b and c). The proliferating cells are small basophilic cells that have a large nuclear to cytoplasm ratio (Figure 2d) and are reminiscent of oval cells.[15] Occasional isolated hepatocytes are observed within the lesions (Figure 2d). It is likely that these hepatocytes are physically isolated by the proliferative epithelial cells, although it is possible that the proliferating cells are differentiating into hepatocytes. Additionally, many dysplastic bile ductular structures were observed within the proliferative areas (Figure 2, b and c, and Figure 3), suggesting that these cells are capable of differentiating into bile ductules.

As mentioned, the severity of the hepatic phenotype varies greatly. Interestingly, the degree of the

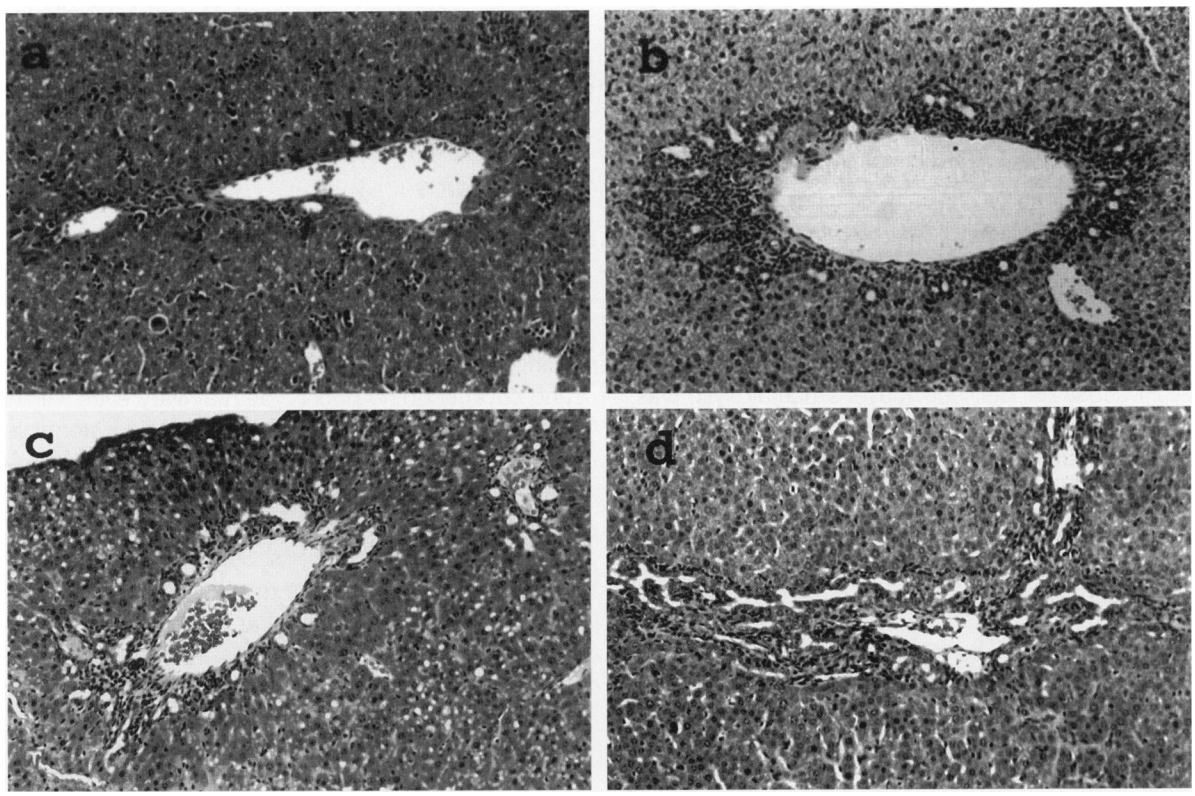

Figure 1. *Developmental time course of lesion progression in the* TgN737Rpw *mouse liver.* **a:** *Photomicrograph of H&E-stained section through a 5-day-old wild-type mouse liver. Note the presence of ductular structures on either side of the portal vein* (**arrows**). **b:** *Section through a 5-day-old* TgN737Rpw *mouse liver with the first indication of proliferating epithelial cells at this time in the midst of persistent extramedullary hematopoiesis. In addition, a number of ductular structures were observed in the portal region.* **c:** *Section through a 10-day-old mouse liver. Proliferating cells have started to expand the portal region, and numerous dysplastic and normal-looking ductular structures were present around the portal vein.* **d:** *Section through a 16-day-old mouse liver. The proliferating epithelial cells were observed within the periportal region in addition to the portal area. Dysplastic ductular structures can be seen within the periportal region. Magnification,* ×*100.*

liver pathology does not seem to correlate with the survival of the mutant animals. What is generally observed in older mice is the proliferation of a biliary epithelial cell population that expanded the portal and periportal region (Figure 2a). In some *TgN737Rpw* mice, the liver lesion progressed to a stage in which much of the liver lobe was filled with anastomosing tortuous bile ductules (Figure 3, a and b). This phenotype was seen infrequently and only in older mice, yet multiple dysplastic ductule structures were always observed in *TgN737Rpw* liver lesions, even at early ages. This may indicate that there was a progressive differentiation toward the ductule structures and that the phenotype shown in Figure 3 may represent an end-stage hepatic phenotype in these mice. The degree of hepatic fibrosis varies greatly depending upon the genetic background upon which the mutation is placed.[1,2] On the FVB/N genetic background that is described here there is little appreciable portal fibrosis (data not shown).

Embryonic and Neonatal Expression of TgN737Rpw *in the Liver*

The expression of wild-type *Tg737* mRNA in the developing embryo and in the adult mouse has been studied previously and was shown to be expressed in all tissues examined (J. Moyer et al, paper in preparation). In mutant mice, the expression of the primary 3.2-kb transcript was shown to be disrupted in all tissues analyzed, including the liver.[1,2] To understand better the role that this gene has in the development of the liver phenotype, the expression of the *Tg737* gene during embryonic and neonatal liver development in wild-type mice was examined by RNAse protection (Figure 4). Utilizing a probe that protects a 400-bp fragment of *Tg737* mRNA, we detected low levels of *Tg737* gene expression at all time points examined. The levels of *Tg737* expression were much lower than that observed for the β-actin internal control. No apparent differences in

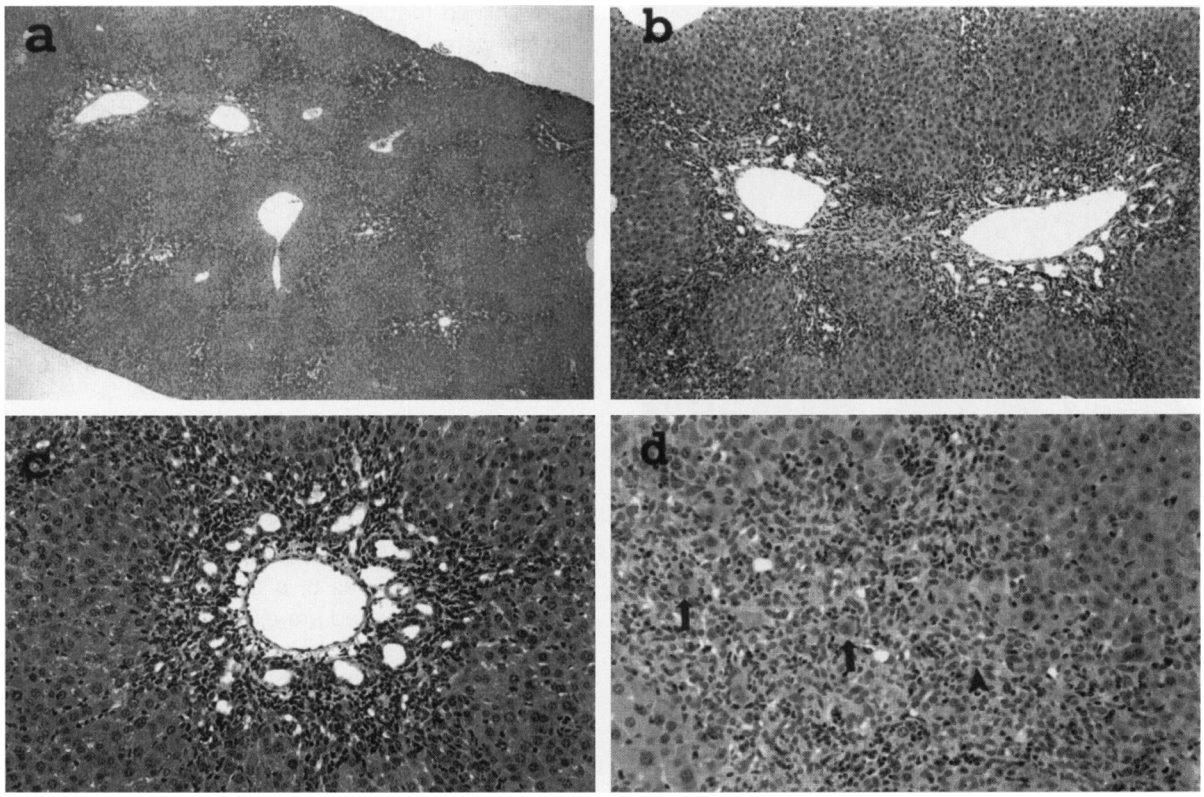

Figure 2. *Adult* TgN737Rpw *mutant liver.* a: *Photomicrograph of H&E-stained section through an adult (43-day-old) mutant liver. Proliferating biliary epithelial cells can be seen expanding the portal region and delineating the periportal region. Magnification, ×40.* b: *Photomicrograph (×100) of portal region in adult mutant liver. Numerous dysplastic ductules occur within the region expanded by the proliferating cells. Additionally, limited disruption of the hepatic parenchyma is observed.* c: *Photomicrograph (×200) of portal region within an adult mutant mouse liver. The portal vein can be seen surrounded by multiple dysplastic ductules.* d: *Photomicrograph (×400) of proliferating epithelial cells. The cells are small in nature with a scant cytoplasm and ovoid nucleus* (arrowhead). *Isolated hepatocytes are occasionally observed within the lesions* (arrows).

the level of *Tg737* mRNA expression were detected during embryonic or neonatal development, indicating that this gene is expressed at constant levels during liver development. Similar levels of expression were also detected in the adult liver (data not shown).

Immunohistochemistry

The proliferating cells within the livers of *TgN737Rpw* mice were further characterized by studying the expression of cell markers. Sections of livers isolated from mutant mice were stained with an antibody that recognized CKs of 56 and 64 kd (Figure 5a) expressed in developing and mature biliary cells.[38] A similar staining pattern was observed with the lectin DBA that recognized mature bile ductular structures (Figure 5b). Within these sections, the ductular structures present within the areas of cellular proliferation were recognized, indicating that these structures exhibit mature biliary characteristics. The cells that formed the lesions were not recognized by DBA, indicating that these cells were not mature biliary epithelial cells. Interestingly, the lectin SJA (Figure 5c), which recognizes biliary cells, detected only a population of cells directly surrounding the portal triad.

An antibody directed against AFP recognized a similar subset of cells as those detected by SJA (Figure 5d). As with SJA, the cells recognized by the AFP antibody were generally located close to the portal region, whereas those cells within the periportal region were not recognized by this antibody. This result indicated that some cells within the lesions are expressing a protein normally found in immature hepatocytes and suggested that the lesions formed by the cells contained a population of cells in different states of differentiation. This result was corroborated by the use of an antibody (A6) raised against dipin-induced mouse oval cells.[22,37] In mutant livers, this antibody detected an antigen present on the ductular structures that formed within the lesions (Figure 5e). Additionally, many, but not all, of the

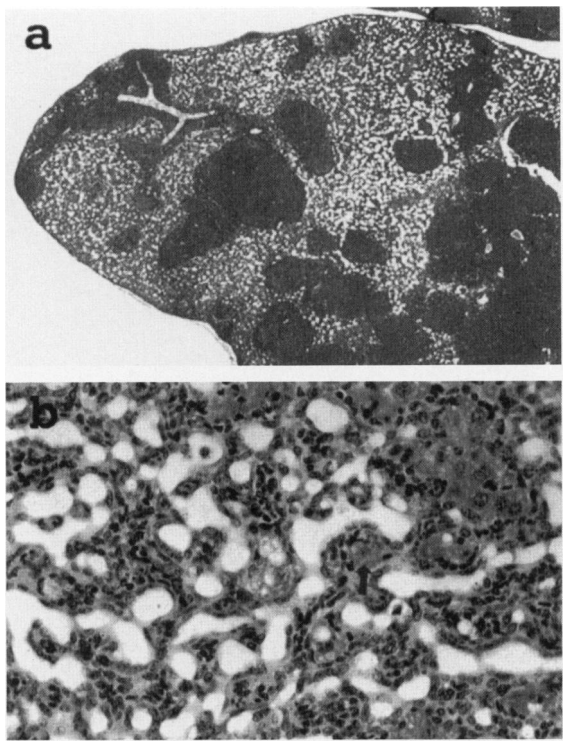

Figure 3. *Anastomosing tortuous bile ductules within adult* TgN737Rpw *livers.* a: *Photomicrograph (×40) of H&E-stained section through an adult mutant liver showing the extreme hyperplasia of dysplastic ductules, where much of the hepatic parenchyma is replaced by the ductular structures.* b: *Photomicrograph (×400) of dysplastic ductular structures within the mutant liver showing the flattened elongated appearance of the ductular structures similar to that observed in children with ARPKD. Isolated hepatocytes are also observed within these regions* (arrows).

cells present within the lesions were recognized by this antibody.

Electron Microscopy

The pleomorphism in the proliferating cells observed by light microscopy was also evident in the ultrastructure (Figure 6). Cells that formed primitive ducts had features of biliary epithelial cells. The cells had small oval nuclei with prominent nucleoli. Small microvilli lined the luminal surface, numerous desmosomes were present between adjacent cells, and an incomplete basement membrane was present in some of the more differentiated ductules (Figure 6a). Mitochondria and endoplasmic reticulum were small and sparse.

The less differentiated oval cells had irregular oval-shaped nuclei with coarsely clumped peripheral chromatin and irregular cell borders. Some microvilli were present on the surfaces of some of the cells. Desmosomes were rare, basement membranes were absent, and the cytoplasm contained few organelles (Figures 6, b and c).

Transitional cells with features of immature hepatocytes were also present in the lesions. These small hepatocyte-like cells had round regular nuclei, numerous mitochondria, moderate endoplasmic reticulum, and occasional peroxisomes and lacked any basal lamina (Figures 6, b and d). Whether these cells represent altered pre-existing hepatocytes, the progeny of oval cells, or immature hepatocytes derived from the proliferation of pre-existing hepatocytes is unknown.

Liver Chemistry of TgN737Rpw *Mutant Mice*

To characterize further the pathophysiology of the mutant phenotype and to gain insight into the role that the mutant liver histopathology played in the clinical manifestation of the *TgN737Rpw* phenotype, we compared serum chemistry from mutant, wild-type, and heterozygous mice (Table 1). The ability to isolate sufficient quantities of serum from mutant mice was complicated by severe growth retardation and early death. This obstacle was overcome by utilizing *TgN737Rpw* animals in which the kidney but not the liver phenotype was corrected by transgenic technology.[39] These partially rescued mutant mice were generated by expressing the predominant 3.2-kb *Tg737* cDNA, un-

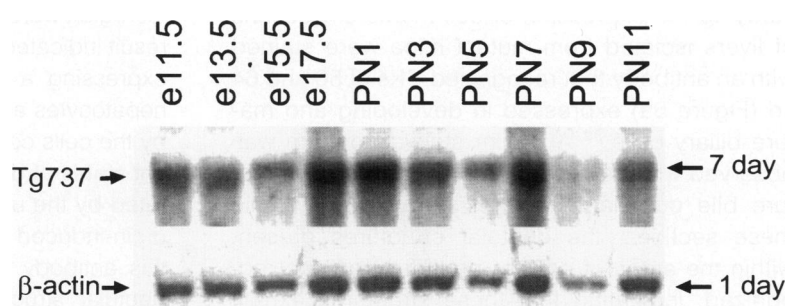

Figure 4. *Developmental expression of* Tg737 *in the mouse liver. Shown is a RNAse protection assay on RNA isolated from the livers of embryonic (e) days 11.5 to 17.5 and postnatal (PN) days 1 to 11 wild-type mice. The* Tg737 *riboprobe and control (β-actin) riboprobe were included within the same reaction. The exposure time of each signal is indicated to the right of the figure.*

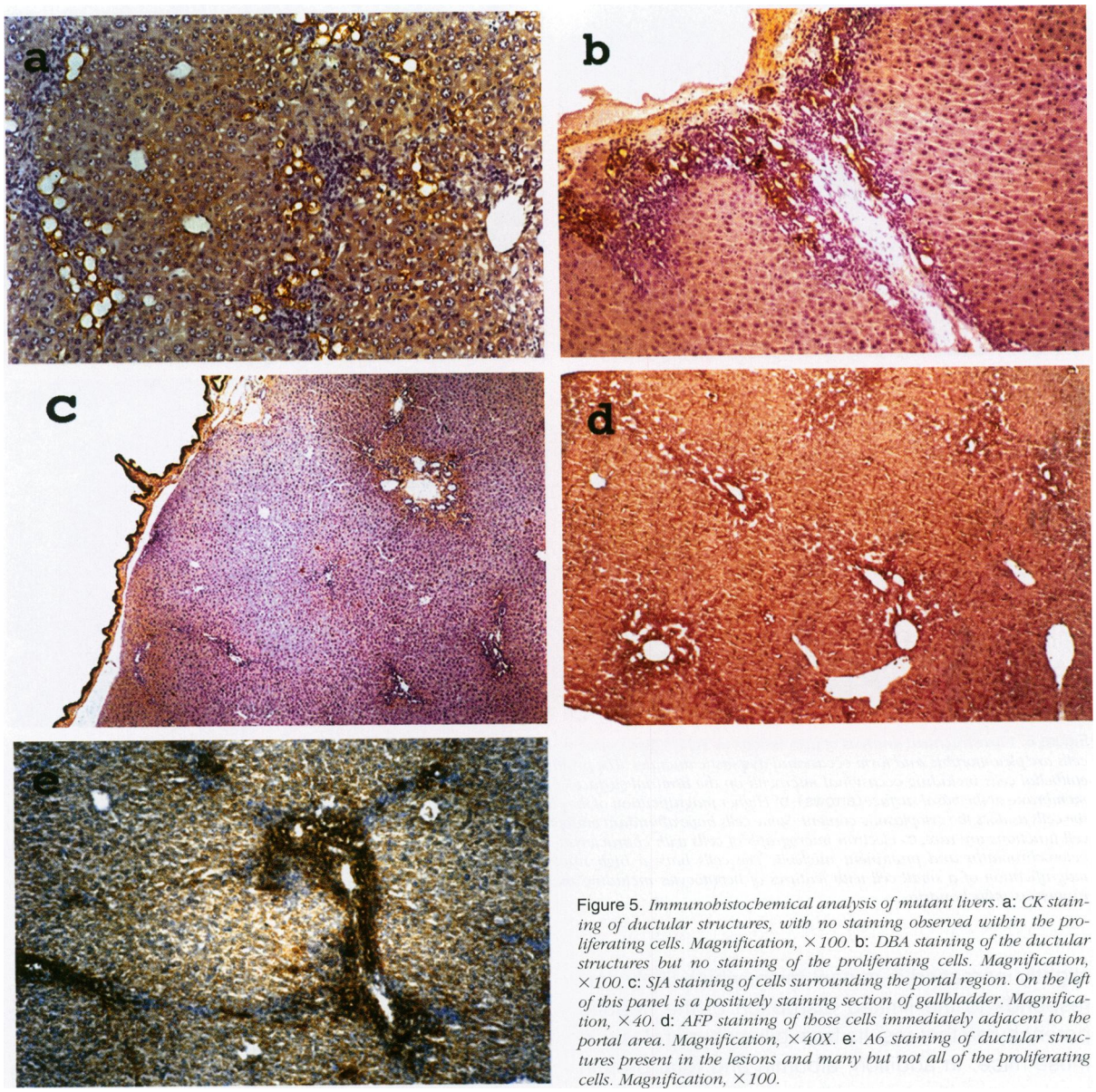

Figure 5. *Immunohistochemical analysis of mutant livers.* a: *CK staining of ductular structures, with no staining observed within the proliferating cells. Magnification, ×100.* b: *DBA staining of the ductular structures but no staining of the proliferating cells. Magnification, ×100.* c: *SJA staining of cells surrounding the portal region. On the left of this panel is a positively staining section of gallbladder. Magnification, ×40.* d: *AFP staining of those cells immediately adjacent to the portal area. Magnification, ×40X.* e: *A6 staining of ductular structures present in the lesions and many but not all of the proliferating cells. Magnification, ×100.*

der control of the human β-actin promoter, in the mutant *TgN737Rpw* background. Mutant mice carrying the rescue transgene were generally larger and longer lived than the original mutant mice, thereby allowing us to isolate adequate serum volumes to perform these assays. No significant differences in liver chemistry or phenotype were observed between mutant and partially rescued *TgN737Rpw* mice and they were treated as a single population for this study.

Alkaline phosphatase, a measure of biliary abnormalities, was markedly elevated in the serum of mutant mice when compared with control littermates, indicating that the biliary system is affected by this mutation. Likewise, the elevation in bile acids is also attributed to the failure of the biliary system to properly secrete bile into the digestive system. Although this increase could be due to an increased secretion of bile acids by hepatocytes, this latter possibility is unlikely as hepatocellular function does not appear to be altered in these mice (see below). A significant increase in cholesterol level was also observed in these mice, suggesting that the mutation affected cholesterol homeostasis.

In contrast, hepatocellular enzymes and molecules that reflect liver function and hepatic parenchyma integrity were normal to mildly elevated in mutant mice. Both AST and ALT levels were

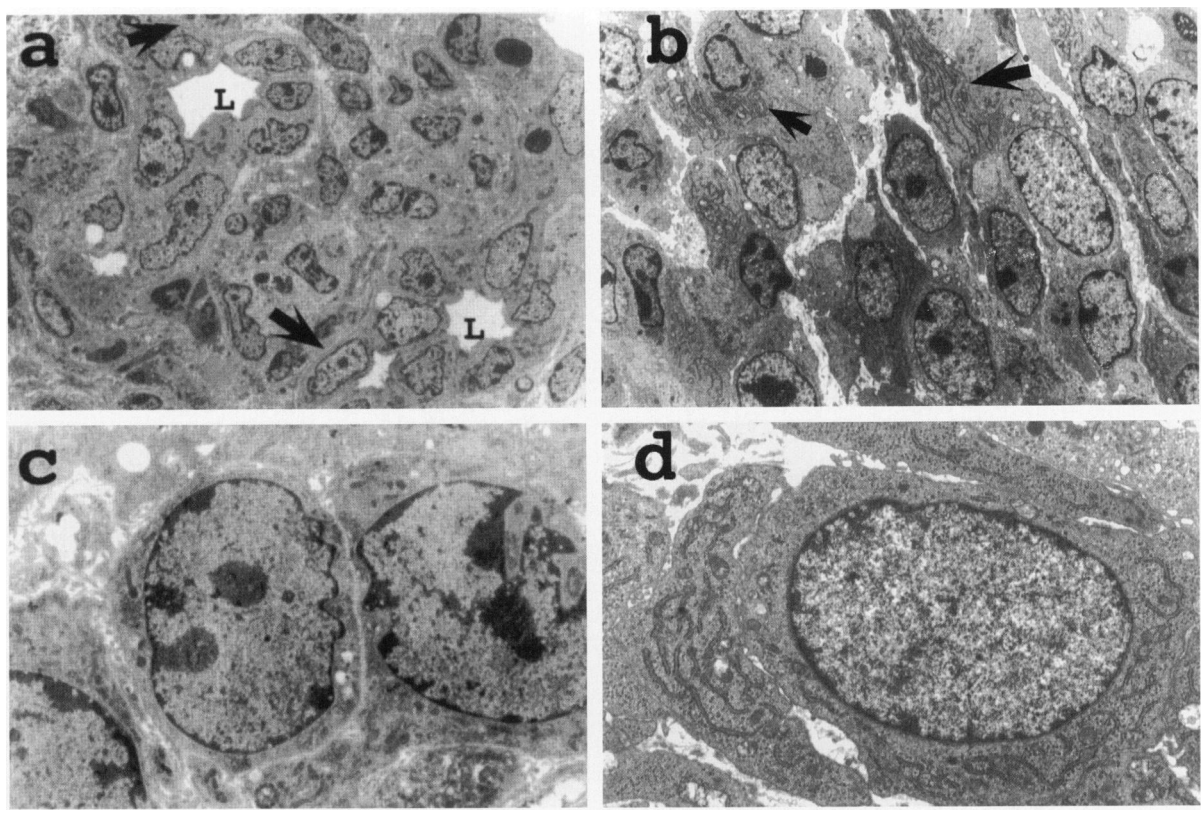

Figure 6. *Ultrastructural analysis of liver lesions in* TgN737Rpw *mice.* a: *Low magnification electron micrograph of proliferating cells. The epithelial cells are pleiomorphic and form occasional dysplastic ductules. The primitive ductules are lined by epithelial cells with some characteristics of biliary epithelial cells including occasional microvilli on the luminal surface (L), numerous desmosomes near the apical surface, and a patchy basement membrane at the basal surface* (arrows). b: *Higher magnification of the pleiomorphic proliferating cells. The nuclear/cytoplasmic ratio varies between the cells as does the cytoplasmic content. Some cells have abundant endoplasmic reticulum* (arrow). *Collagen is present in the intercellular space, and cell junctions are rare.* c: *Electron micrograph of cells with characteristics of oval cells including an oval, slightly irregular nucleus with peripheral heterochromatin and prominent nucleoli. The cells have a high nuclear/cytoplasmic ratio and the cytoplasm have scant organelles.* d: *High magnification of a small cell with features of hepatocytes including moderate amounts of cytoplasm with abundant endoplasmic reticulum and numerous mitochondria.*

slightly increased in mutant mice, which likely corresponds to the limited disruption of the hepatic parenchyma that results from biliary hyperplasia in these mice. In addition, albumin and total protein levels were within normal limits in the mutant mice, suggesting that hepatocellular function was adequate.

Discussion

The pathology of the liver in *TgN737Rpw*-FVB/N mice involves a unique lesion associated with epithelial cell proliferation and dysplastic ductule formation that is associated with a polycystic kidney phenotype. The *TgN737Rpw* model was generated by an insertional mutation resulting from the integration of a transgene into a gene on chromosome 14. The expression of this gene, *Tg737,* was shown previously to be disrupted in all tissues analyzed from mutant animals, including the liver. The expression of *Tg737* is detected in wild-type livers at all time points observed, including the time during which the hepatic phenotype first became apparent in mutant animals. The expression of *Tg737* at the time during which the mutant phenotype was first detected (5 days old) indicated that the loss of *Tg737* gene expression may play a primary role in the etiology of this phenotype. Whether this abnormality was intrinsic to the proliferating biliary cells or whether it resulted from an earlier developmental abnormality caused by lack of *Tg737* expression remains to be determined.

The liver pathology in these mutant animals was characterized by a defect in the intrahepatic biliary tract. This defect included the proliferation of an epithelial cell population with characteristics of oval cells and the formation of tortuous anastomosing bile-ductule-like structures within the proliferative lesions. Additionally, immunohistochemistry and serum chemistry performed on *TgN737Rpw* mutant mice indicated that the defect was of biliary origin;

Table 1. *Serum Chemistry of* TgN737Rpw *Mutant Mice*

Chemistry panel	Wild-type/heterozygous ± SD (n)	Mutant ± SD (n)
Albumin (g/dl)	2.92 ± 0.23 (11)	2.54 ± 0.76 (14)
Alkaline phosphatase (IU/L)	93.45 ± 35.27 (11)	2473 ± 1123 (14)
AST (IU/L)	86.18 ± 65.15 (11)	321.5 ± 166.23 (14)
ALT (IU/L)	57.91 ± 35.86 (11)	160 ± 72.94 (14)
BUN (mg/dl)	36.34 ± 18.75 (11)	29.0 ± 17.07 (14)
Calcium (mg/dl)	9.13 ± 0.99 (8)	9.44 ± 1.34 (7)
Creatinine (mg/dl)	0.44 ± 0.11 (11)	0.38 ± 0.21 (14)
Glucose (mg/dl)	145.88 ± 34.37 (8)	148.29 ± 27.54 (7)
Cholesterol (mg/dl)	106.38 ± 23.45 (8)	551.57 ± 468.71 (7)
Phosphorous (mg/dl)	7.94 ± 4.05 (8)	7.49 ± 1.04 (7)
Total protein (g/dl)	4.69 ± 0.49 (8)	4.76 ± 0.73 (7)
Total bilirubin (mg/dl)	0.38 ± 0.23 (11)	1.51 ± 1.51 (14)
Globulin (g/dl)	1.80 ± 0.3 (8)	2.11 ± 0.45 (7)
A/G	1.63 ± 0.2 (8)	1.28 ± 0.15 (7)
Bile acids (Umol/L)	7.50 ± 7.89 (6)	601.66 ± 483.72 (5)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; BUN, blood urea nitrogen; A/G, albumin/globulin.

the slight elevation in hepatic leakage enzymes noted in mutant mice likely corresponds to the limited disruption of the hepatic parenchyma by the proliferating cells. Furthermore, tests for hepatocellular function were not significantly altered, indicating that hepatocytes were relatively unaffected in this model. Instead, the marked increase in alkaline phosphatase and bile acids suggested that the primary liver defect in *TgN737Rpw* mice was of biliary origin.

Interestingly, the hepatic phenotype in mutant mice closely resembled that observed in rat models of oval cell proliferation.[40–42] For instance, the cellular proliferation observed in mutant livers emanated from the portal region and eventually expanded the portal area and into the periportal space. A similar event has been described in rats maintained on a choline-deficient diet and fed the hepatocarcinogen acetylaminofluorine.[43] In *TgN737Rpw* mice, the proliferating cells disrupted the limiting plate and were detected within the hepatic parenchyma, where they isolated individual hepatocytes. However, we do not see a large infiltration of these cells into the liver lobule as reported in the aforementioned models. Likewise, this model appears unique in that a cell population with characteristics similar to those described for oval cells is induced to proliferate without overt damage to the hepatic parenchyma.

In addition to the lesions formed by the proliferation of a biliary epithelial cell population, we observed the formation of ductular structures within the proliferative zones. These structures varied in morphology from being dysplastic to resembling normal bile ductules. Generally, a greater degree of ductule proliferation was observed in older mutant animals, although dysplastic bile ductules were observed at the earliest appearance of the phenotype. The fact that ductular structures formed within the proliferative zones and that with time these structures appeared to replace the proliferating cells suggests that the proliferating epithelial cells were capable of differentiating down the biliary pathway.

The ultrastructural characterization of cells within the lesions indicated that many cells had characteristics similar to those described for oval cells. These included an ovoid nucleus with peripheral heterochromatin, the absence of basement membranes, and an organelle-sparse cytoplasm. In addition to oval cells, biliary epithelial cells and immature hepatocytes were observed within the lesions. Whether the small hepatocytes observed within the proliferative lesions are transitional cells that arose from the differentiation of oval cells remains to be determined.

Few antibodies have been raised to mouse oval cells. Among those that have been is the A6 antibody generated against oval cells in the dipin-induced murine hepatocarcinogenesis model.[22,37] We used this antibody to study the expression of the A6 epitope in *TgN737Rpw* mutant livers. The strongest reactivity was noted on the ductular structures that formed within the proliferative lesions. Reactivity was detected in many, but not all, of the proliferating cells. There appeared to be a gradient of reactivity to this antibody, with the strongest reactivity observed in and around the portal spaces with diminishing or no reactivity observed in cells in the periportal region.

In addition to the A6 antigen, oval cells have been reported to express other markers including some found in hepatocytes and in biliary epithelial cells. The expression of AFP has been visualized in the D-galactosamine model by *in situ* hybridization analysis and was shown to be expressed in a subset of

oval cells.[15] Using immunohistochemistry, we detected AFP expression primarily within cells in the portal regions of mutant livers. Little, if any, immunoreactivity was detected in the epithelial cells within the periportal region, including those regions in which A6 reactivity was generally detected, indicating that AFP-positive cells make up a subset of A6-positive cells. The lectin SJA, which recognized biliary epithelial cells, also recognized a population of cells similar in location to those detected by the AFP antibody.

Taken together, the immunohistological data indicated that the liver lesions in *TgN737Rpw* mice were composed of a population of cells that exhibited a differential expression of markers including some that are present in oval cells, such as A6 and AFP. Furthermore, there appears to be a subset of A6-positive cells that express both a hepatocellular marker (AFP) and a marker present on biliary cells (SJA). The expression of hepatocellular and biliary markers has been described in oval cells,[15,42] and the presence of these markers in a cell type surrounding the portal triad in *TgN737Rpw* mutant mice provided additional evidence for the presence of oval cells within these lesions. The data also suggested that the lesions were composed of cells at various stages of differentiation.

Role of the Tg737 *gene*

The fact that this mouse model was generated by transgene insertional mutagenesis allowed us to identify a gene the expression of which was disrupted in homozygous mutant animals. Analysis of the predicted amino acid sequence of the *Tg737* cDNA revealed that the protein contained 10 copies of a motif, termed the TPR motif, that has been found in many proteins involved in regulating the cell cycle in organisms as diverse as yeast and humans. Given that the hallmark of this mutation is the proliferation of epithelial cells, not only in the liver but also in the kidney and pancreas, it is suggested that the Tg737 protein might function in the regulation of cellular proliferation. Given the homology of Tg737 to other TPR-containing proteins involved in cellular proliferation,[3] it might follow that Tg737 acts directly within the cell cycle. An alternative idea, yet not mutually exclusive, would be that Tg737 is involved in the differentiation of the proliferating cell types. A failure of the affected cell type to respond properly to differentiation signals could also lead to a proliferative phenotype. Interestingly, the affected cell types both in the liver (oval cells) and in the kidney (principal cells) display an immature phenotype, an observation that may support the role of Tg737 in differentiation. A direct test of both of these hypotheses will be possible by the expression of *Tg737* in cells isolated from mutant livers.

The TgN737Rpw *Mouse as a Model to Study Liver Disease in ARPKD*

Children with ARPKD are invariably inflicted with liver abnormalities in conjunction with polycystic kidneys. This liver disease, referred to as congenital hepatic fibrosis, is characterized by biliary hyperplasia, ductular hyperplasia and dysgenesis, and varying degrees of fibrosis.[4,5] One of the difficulties in treating children with ARPKD is that, even when the kidney defect is corrected though transplantation or dialysis, these children will be compromised by the liver defect.[44]

To date, the *TgN737Rpw* mouse is the only PKD model from which a mutated gene has been cloned. This allows one to study the involvement of Tg737 in the hepatorenal pathology. Moreover, this is one of the few animal models available that has the dual hepatorenal pathology associated with ARPKD, making it a useful model system to study the pathophysiology involved in the development of these abnormalities. Like ARPKD, the biliary dysgenesis in the *TgN737Rpw* mouse included the proliferation of ductular structures. The shape of these structures varied widely, and often they appeared enlarged and flattened. In addition, the degree of portal and periportal fibrosis was variable and was profoundly affected by the genetic background upon which the mutation was placed.

Other human abnormalities exist that have a renal and hepatic pathology similar to that observed in the *TgN737Rpw* mouse model. Patients with Meckel-Gruber syndrome exhibit a similar renal pathology and a hepatic pathology that includes varying degrees of bile duct proliferation and dilation as well as portal fibrosis, similar to that seen in the *TgN737Rpw* mouse.[45,46] In addition, the *TgN737Rpw* model exhibits other pleiotropic phenotypes similar to those observed in Meckel-Gruber syndrome, such as polydactyly. Ivemark's syndrome, in addition to exhibiting a similar hepatorenal dysplasia, includes a pancreatic dysplasia that is an additional phenotype observed in the *TgN737Rpw* model.[47] The involvement of the *Tg737* gene in any of these human diseases remains to be determined. Regardless, the *TgN737Rpw* mouse remains a model system in which the pathophysiology of hepatorenal abnormalities can be studied, therapeutic strategies tested, and modifying genes identified.

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