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Short Communication

Isolation and Characterization of Liver Epithelial Cell Lines from Wild-Type and Mutant *TgN737Rpw* Mice

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The* Tg737 *gene encodes a tetratricopeptide repeat containing protein that, when disrupted in* TgN737Rpw *mutant mice, results in pleiotropic phenotypes that include the proliferation of epithelial cells. In the kidney and liver, this causes a phenotype that resembles autosomal recessive polycystic kidney disease. In the liver, the affected epithelial cells morphologically and immunologically resemble oval cells. Here we describe the isolation, culture, and characterization of epithelial cell lines derived from the livers of wild-type, heterozygous, and homozygous* TgN737Rpw *mice. Essentially homogeneous cell cultures were established and the expression of liver markers was examined by reverse transcriptase polymerase chain reaction and by immunohistochemistry. All of the cell lines reacted to the A6 antibody that was raised against mouse oval cells and expressed markers seen in oval cells. Cells transplanted into the interscapular fat pads of isogenic mice formed well defined ductular structures. Furthermore, in transfection experiments, we have demonstrated the involvement of* Tg737 *in cellular proliferation. (Am J Pathol 1997, 150:1189–1197)

The *TgN737Rpw* mouse exhibits a phenotype that closely resembles the pathology observed in patients with autosomal recessive polycystic kidney disease (ARPKD).[1, 2] This phenotype includes polycystic kidneys, biliary hyperplasia, and the formation of dysplastic ductular structures within the liver. The unique aspect of the *TgN737Rpw* model is that the gene responsible for the syndrome has been identified. The characterization of this gene will lead to a better understanding of the molecular events involved in generating the pathology in *TgN737Rpw* mice, which may help to resolve the hepatic and kidney disease observed in ARPKD patients.

Due to the ability to successfully treat the renal pathology in ARPKD patients through kidney transplantation and dialysis, the hepatic defect in these patients is of increased significance as the liver deficiencies can lead to complications that may compromise long-term survival.[3] It is therefore critical to understand the liver defect associated with this dis-

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ease. One of the hallmarks of ARPKD and other diseases involving dual hepatorenal pathology is the proliferation of epithelial cells in the affected organs.[4-6] In the liver of *TgN737Rpw* mice, loss of the Tg737 gene product results in the proliferation of a biliary epithelial cell population with characteristics similar to that described for oval cells.[7,8] These cells then appear to give rise to dysplastic ductular structures that are similar to those observed in ARPKD patients.[9]

To understand better the proliferating epithelial cells in *TgN737Rpw* mice, we have isolated cell lines from mutant and heterozygous *TgN737Rpw* mouse livers as well as from wild-type mouse livers. These cells provide a tool to study the function of the *Tg737* gene product and may provide a valuable reagent for studying the role of this cell type in liver carcinogenesis and organogenesis. Gene expression and immunohistochemistry indicate that these cells exhibit characteristics similar to those described for oval cells.[7] Furthermore, the transfection of a mutant cell line with the wild-type *Tg737* cDNA slows the proliferation of this cell type.

Materials and Methods

Cell Line Derivation and Culture

The liver cell lines described in this paper were established by the use of long-term culture conditions as described.[10,11] Briefly, livers were isolated from wild-type, heterozygous or *TgN737Rpw* mice,[1,2] minced into small pieces, washed in phosphate-buffered saline, and digested with 0.25% collagenase at 37°C for 30 minutes. The samples were centrifuged at 100 × *g* for 5 minutes and then washed once in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco). The cell pellet was resuspended in DMEM/F12 plus 10% fetal calf serum (FCS) and plated in a 25-cm tissue culture flask (Falcon, Franklin Lakes, NJ). Cells were allowed to adhere overnight, and the next morning the flasks were washed and new medium was added. The cultures were fed DMEM/F12 plus 10% FCS every 3 to 4 days and were passaged once per month. After the majority of cells had undergone cell crisis, an essentially homogeneous culture was established. All cell lines described were established from different mice. The established cell lines were treated normally and were passaged at confluence and maintained on DMEM/F12 plus 10% FCS for the experiments described within this paper. Clones were established by limiting dilution.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis of Gene Expression

Total RNA was isolated with RNA STAT-60, following the manufacturer's instructions (Tel-Test "B", Friendswood, TX). Reverse transcription (RT) reactions were conducted using random hexamer priming on total RNA as described elsewhere.[12] First-strand cDNAs were suspended in 100 μl of dH20 and stored at −80°C until use. PCR reactions used 2 μl of the first-strand cDNA mixture. PCR was conducted using the following conditions: 45 seconds at 93°C, 30 seconds at 60°C, and 45 seconds at 72°C. For PCR, the following primer pairs were used: (listed in the order of primer set forward primer, reverse primer): Tg737, AGATGTTCCTCAGGCACCAT, ACAGCACAAACCCATCCTCA; α-fetoprotein (AFP), TCCCACCCTTCCAGTTTCCA, CACATCCAGGGCCAGCTTCT; albumin, TGGCTGACTGCTGTGCAAAAC, AACGTGCCCAGGAAGACATCC; c-Met, AGATGAACGTGAACATGAAG, CTAATGAGTTGATCATCATAG; c-Kit, AACTTTTCCTGGTTGGCCTT, CACGTTTTTGATGGTGATGC; hepatocyte growth factor, CCATGAATTTGACCTCTATG, ACTGAGGAATGTCACAGACT; Bc12, GTAAATTGCCGAGAAGAAGGG, CCAGATTGGGTCCTCACACT; p53, AGAGACCGCCGTACAGAAGA, ATGGCGGGAAGTAGACTGG; c-Myc, TCTCCACTCACCAGCACAAC, TGAGGATCACTACCTTGGGG; connexin 26, CGTCTGGTGAAATGCAACGC, CTTGGGAACTGCTGGTGGCT; connexin 32, ATTTTTTCCCCATCTCCCAC, CGGAACACCACACTGATGAC; connexin 43, GCTAGGCGGCAAAAGTAGG, GCCATGTTGAGGACTTGGAT; mdr2, CATGAAACTGCCCCAGAAAT, ATCGGTGAGCTATCACAATGG; epidermal growth factor receptor (EGF-R), TCCCAGAAGGTGAGAAAGTA, TCAGACAGATGCCCAGGAG; and HPRT, CCTGCTGGATTACATTAAAGCACTG, GTCAAGGGCATATCCAACAACAAAC. Oligonucleotides were synthesized on an Applied Biosystems (Foster City, CA) PCR-MATE model 391.

Immunohistochemical Analysis

Immunohistochemical analysis using the A6 rat monoclonal antibody raised against mouse oval cells[13,14] (kindly provided by Dr. N. Engelhardt, Institute of Carcinogenesis, Cancer Research Center, Moscow, Russia) and the BrdU-specific rat monoclonal antibody (Sera-Lab, Sussex, UK) (nonspecific control) was performed as described.[8]

Interscapular Fat Pad Injections

Interscapular fat pad injection of BroC2 and HL617 cells was conducted as previously described in the rat.[15] Cells were allowed to incubate for 1 month, at which time the mice were sacrificed and the fat pads isolated. The fat pads were fixed in buffered formalin overnight, sectioned, and stained with hematoxylin and eosin (H&E).

Cellular Proliferation Assay

HL617 cells were co-transfected with a *Tg737* expression construct that used the human β-actin promoter to drive expression of the *Tg737* cDNA and a phosphoglycerate kinase-neomycin (PGK-Neo) expression construct. Control cells contained only PGK-Neo. Cells were transfected with 2 μg of DNA and 10 μl of lipofectin, following the manufacturer's instructions (Gibco BRL, Grand Island, NY), and stable cell lines were selected in 300 μg of G418 (Gibco). Cells were plated at various viable cell densities in 96-well flat-bottom plates (replicates of four). The total volume was 0.2 ml, and at various time points 1.0 μCi of [^{3}H]thymidine (Amersham, Arlington Heights, IL) was added per well. After 18 hours at 37°C, the cells were washed and label incorporation determined on a Packard Matrix 96 beta counter. The data presented are for cells plated at 12,500 cells/well. Similar results were obtained with other dilutions.

Results

Isolation of Liver Cell Lines

To better characterize the defect in *TgN737Rpw* mice, we established cell lines from the livers of wild-type, heterozygous, and homozygous *TgN737Rpw* mice using long-term culture conditions to establish essentially homogeneous populations of liver epithelial cells.[10, 11] Liver A (LA) cells were isolated from wild-type mice, liver 2 (L2) cells from heterozygotes, and HL617, BroC2, and BroF2 cells were derived from homozygous *TgN737Rpw* mouse livers. The LA, L2, and BroF2 cells were all generated from livers isolated from FVB/N mice whereas the HL617 and BroC2 cells were isolated from the C3H genetic background.

The cells within the homogeneous cultures exhibited an epithelial morphology. When these cells were passaged and plated, they formed colonies that would expand with time. At confluence and within the colonies, the cells exhibited a cobblestone-like appearance. Additionally, long filamentous extensions were seen protruding from some of the cells and making contact with other cells. Occasionally, large flattened cells that were binucleated were observed in both the cell population and in clonally derived cells. The nature of these larger cells has not been determined.

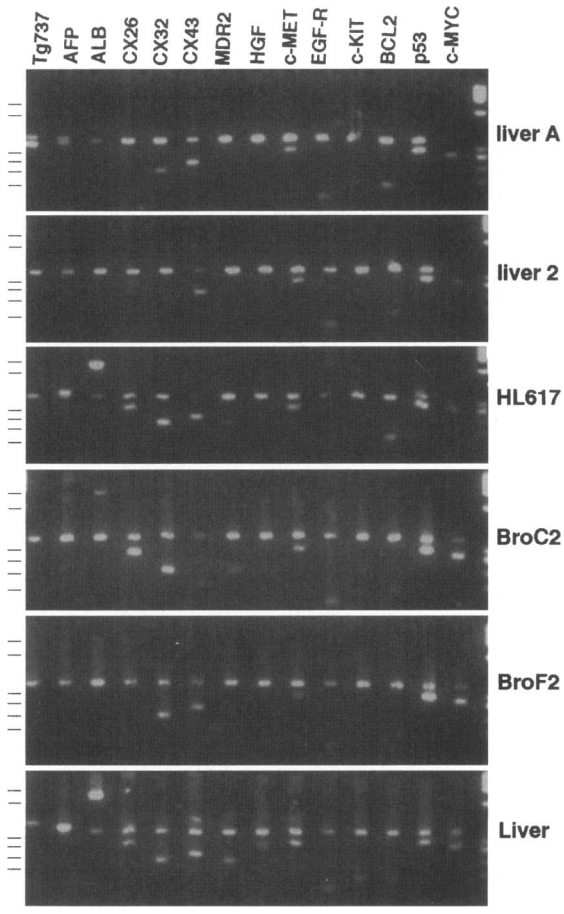

Figure 1. *Gene expression in the liver cell lines. RT-PCR was used to analyze gene expression in the isolated cell lines. RNA was subjected to reverse transcription and PCR using the indicated primer set and HPRT primers as an internal control (325 bp). Shown in the* first lane *of each primer set is the experimental sample that was reverse transcribed. The* second lane *represents control RNA that was not reverse transcribed. Primers and the expected size fragments generated by PCR are described in Materials and Methods. The* bottom panel *represents total liver RNA isolated from wild-type FVB/N livers and subjected to RT-PCR, thereby providing a positive control for each primer set. Shown to the left of each panel are DNA size markers (top to bottom: 872, 603, 310, 281, 234, and 194 bp).*

Marker Expression in Liver Cell Lines

RT-PCR was used to detect gene expression for a number of genes that have been well characterized in various liver cell lineages (Figure 1 and Table 1). These markers include those expressed in hepatocytes (AFP, albumin, connexins 26 and 32, c-*met*,

Table 1. *Marker Expression in Liver Cell Lines*

	Liver A	Liver 2	HL617	BroC2	BroF2
Tg737	+	+	−	−	−
AFP	+	−	+	−	−
ALB	−	−	+	+	−
cx26	−	+	+	+	−
cx32	+	+	+	+	+
cx43	+	+	+	−	+
mdr2	−	−	+	+	−
HGF	−	−	−	−	−
c-*met*	+	+	+	+	+
EGF-R	+	+	+	+	+
c-*kit*	−	−	−	−	−
Bc12	+	+	+	+	+
p53	+	+	+	+	+
c-*myc*	+	+	+	+	+
A6	+	+	+	+	+

Tg737 expression was detected by RNAse protection only. ALB, albumin; cx26, connexin 26; cx32, connexin 32; cx43, connexin 43; *mdr2*, multi-drug resistance gene 2; HGF, hepatocyte growth factor.

and *mdr2*) and those expressed in oval cells (AFP, connexin 43, and c-*kit*).[16–18] As the *TgN737Rpw* liver phenotype suggested that growth control was altered, a number of genes implicated in this process were examined, including the EGF-R, c-*met*, *bcl2*, p53, and c-*myc*.

The expression of the genes involved in regulating cell proliferation and growth (EGF-R, c-*met*, *bcl2*, p53, and c-*myc*) was detected in all the cell lines. Although no overt differences in the expression of these regulatory genes were detected between wild-type and mutant cell lines, the RT-PCR assay was not performed under quantitative conditions, and a more refined analysis may yield more informative results. c-*kit* expression was not detected in any of the cell lines.

LA cells, which were derived from wild-type mice on the FVB/N genetic background, expressed *Tg737* mRNA. In addition, expression of AFP, connexin 32, and connexin 43 was detected in this cell line.

Interestingly, expression of *Tg737* mRNA could not be detected by RT-PCR in the cell line derived from the liver of a heterozygous *TgN737Rpw* mouse (L2). However, we have been able to detect the expression of a *Tg737* transcript by RNAse protection in this cell line (data not shown). This result may be attributed to the use of RT-PCR primers directed against the 5′ region of the *Tg737* cDNA, whereas the RNAse protection assay used a probe derived from the 3′ end of the cDNA and might indicate that an alternative *Tg737* transcript is expressed in these cells. Neither AFP nor albumin expression were detected in the L2 cell line, although low but detectable levels of connexin 26 and connexin 32 were observed. The expression of connexin 43 was readily detectable in this cell line.

BroF2 cells were isolated from a mutant *TgN737Rpw* liver and therefore do not express *Tg737* mRNA. In addition, these cells do not express the hepatocellular markers, AFP, albumin, connexin 26, and *mdr2*. The expression of connexin 32 and connexin 43 was detected in this cell line.

As expected, the HL617 cell population, derived from a mutant *TgN737Rpw* liver on the C3H genetic background, did not express *Tg737* mRNA. In addition, HL617 cells expressed both AFP and albumin as well as the three connexins. *mdr2* expression was also detected in these cells. The other cell line isolated from a mutant *TgN737Rpw*-C3H liver was the BroC2 cell line. This cell line appeared more differentiated along the hepatocyte lineage as albumin, connexin 26, connexin 32, and *mdr2* expression was detected whereas AFP and connexin 43 mRNA was not detected.

A6 Immunoreactivity of the Liver Cells

The A6 antibody is one of the few antibodies available that were generated against mouse oval cells. In addition to oval cells, this antibody detects biliary epithelium as well as epithelial cell types found in tissues other than the liver.[13, 14] We used this antibody to further characterize liver cell lines isolated from mutant *TgN737Rpw* and wild-type mice. Cells grown in culture were subjected to immunohistochemistry with the A6 antibody (Figure 2). The majority of LA cells were recognized by this antibody with some cells showing increased immunoreactivity when compared with other cells (Figure 2, a and b). Binucleated cells were observed within this population that were not recognized by this antibody (data not shown). Both the heterozygous L2 (Figure 2, c

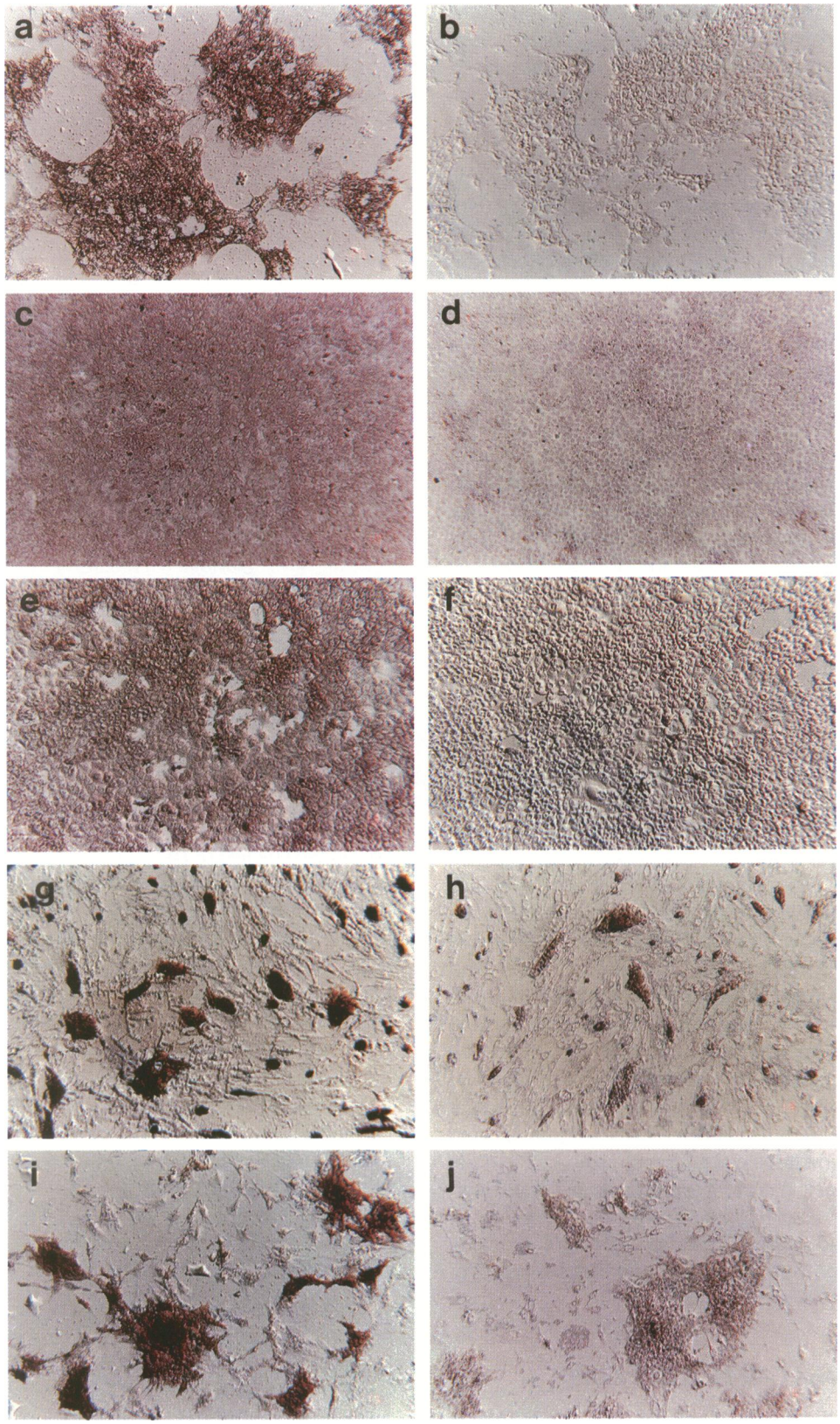

Figure 2. *A6 immunohistochemistry on cultured liver cells. The* left panels (a, c, e, f, g, *and* i) *represent cell lines incubated with the anti-mouse A6 antibody. The* right panels (b, d, f, h, *and* j) *are cultured cells stained with the control antibody.* a *and* b: *Liver A cells.* c *and* d: *Liver 2 cells.* e *and* f: *HL617 cells.* g *and* h: *BroC2 cells.* i *and* j: *BroF2 cells. Magnification, ×100.*

and d) and mutant HL617 (Figure 2, e and f) cell populations exhibited varying degrees of immunoreactivity with the A6 antibody. On the whole, it appeared that fewer HL617 cells were detected by the A6 antibody than were found in the LA or L2 cell lines.

Immunohistochemistry using the A6 antibody was performed on the two mutant cell lines, BroC2 (Figure 2, g and h) and BroF2 (Figure 2, i and j), while they were being derived. Once colonies of cells appeared within the cultures, they were stained. With both the BroC2 and BroF2 cells, only those cells within the colonies that eventually gave rise to the essentially homogeneous cultures were recognized by this antibody. Although increased background staining was observed in the BroC2 cell line, it was clear the BroC2 cell colonies exhibited a far greater degree of immunoreactivity to the A6 antibody than was observed with the control antibody. Other liver cells present within the mixed cultures were not recognized by this antibody; this demonstrated the specificity for the liver cell type that eventually established the BroC2 and BroF2 cell lines.

Growth Characteristics in Interscapular Fat Pads

Initial examination of the *in vivo* differentiation characteristics of these cells was performed by injecting BroC2 cells into interscapular fat pads of isogenic mice. Clonal isolates from the BroC2 cell population were used in these experiments, and similar results were obtained with the HL617 cell line. After approximately 4 weeks, the recipient animals were sacrificed and the interscapular fat pad was isolated and examined histologically for the presence of structures derived from the injected cells. Structures were observed in approximately 50% of the injected mice (8 of 17). Sections obtained from many animals injected with BroC2 clones revealed the presence of ductular structures within the fat pad (Figure 3, a and b). These structures were characterized by the presence of a well defined columnar epithelial cell surrounding a lumen (Figure 3b). These structures were not observed in sham-injected (brain extract) control animals. Nor were we able to detect cells morphologically similar to hepatocytes.

Tg737 *Slows Cellular Proliferation*

Given that the salient feature of the *TgN737Rpw* is the proliferation of epithelial cells, we have examined the effect of *Tg737* expression on cellular proliferation as our first test of *Tg737* function. To examine the ability of *Tg737* to regulate cellular proliferation we have used one of the cell lines (HL617) described herein. HL617 cells were co-transfected with a *Tg737* mammalian expression construct and a PGK-Neo expression construct or with the PGK-Neo vector alone. Cellular proliferation in control and *Tg737* transfected cells was then determined by the incorporation of tritiated thymidine, which is a measure of cellular replication. The results indicate that cells expressing the *Tg737* cDNA proliferate at a slower rate than cells transfected with the control vector alone (Table 2).

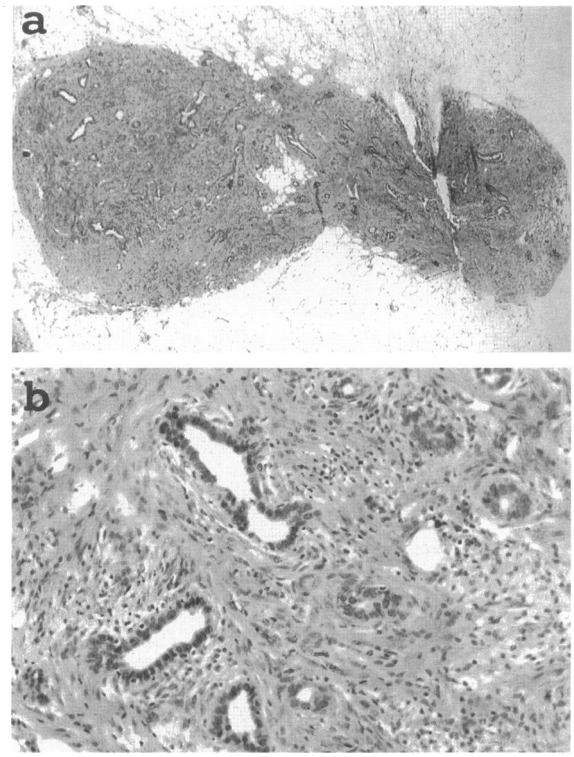

Figure 3. *Interscapular fat pad growth of BroC2 cells. BroC2 clones were injected into interscapular fat pads of isogenic mice. Shown is a representative H&E-stained section of one of the structures obtained with BroC2-D2 cells. Similar structures were observed with two other BroC2 clones injected (A4 and C6).* **a:** *Photomicrograph (×400) showing the structure formed within the fat pad.* **b:** *Increased magnification (×200) of* **a** *showing ductular structures within the reactive tissue.*

Discussion

We described the isolation and characterization of five liver epithelial cell lines. These cell lines were isolated from mice that were wild type, heterozygous, or homozygous for the *TgN737Rpw* allele. The characterization of the cell lines included analyzing the expression of various genes, including markers for hepatocyte differentiation and cellular prolifera-

Table 2. *Regulation of Cellular Proliferation by Tg737*

Cell line	Expression construct	[^{3}H]Thymidine Incorporation (cpm) ± SD		
		6 hours	24 hours	48 hours
HL617	Tg737 and PGK-Neo	1910 ± 695	7268 ± 1680	42502 ± 12187
HL617	PGK-Neo	1269 ± 267	25381 ± 908	133333 ± 9018

Data are presented as the mean from four replicates and were obtained from 12,500 cells plated.

tion. The cell lines derived from the FVB/N genetic background (LA, L2, and BroF2) displayed a gene expression profile similar to that seen in immature liver epithelial cells such as oval cells. This was most evident by the expression of connexin 43 in all three of these cell lines and by the reactivity of these cell lines to the A6 antibody. The expression of the hepatocyte marker, connexin 32, was also detected within all of these cell lines. Whether the expression of connexin 32 in these cell populations indicated that some differentiation toward the hepatic lineage occurred while these cultures were being derived or whether the cell populations were originally composed of different cell types remains to be determined. The ability to clone all of the cell lines described in this paper will make it possible to test these hypotheses.

The cell line derived from a mutant *TgN737Rpw* animal on the FVB/N genetic background, BroF2, did not express many of the markers associated the hepatocellular lineage (ie, AFP, albumin, connexin 26, and *mdr2*). Therefore, it is possible that the *Tg737* gene is involved in the regulation of hepatocellular differentiation in oval cells. Alternatively, the absence of *Tg737* may have no effect upon the differentiation of these cells, and the absence of hepatocellular markers may simply be a result of tissue culture conditions. These hypotheses can now be tested directly as the *Tg737* cDNA has been cloned and can be transfected into the BroF2 cell line and their ability to differentiate with and without *Tg737* expression can be followed.

Cell lines isolated from *TgN737Rpw* mutant mice on the C3H genetic background (HL617 and BroC2) exhibited many hepatocellular characteristics. These included the expression of albumin, connexin 26, connexin 32, and *mdr2* in both cell lines. *mdr2* is present on the bile canicular surface of hepatocytes, and mice deficient for the *mdr2* gene exhibit a similar phenotype of biliary hyperplasia as observed in *TgN737Rpw* mice.[19, 20] The expression of *mdr2* within this mutant cell line indicated that the liver pathology within *TgN737Rpw* mice does not result from the absence of *mdr2* expression. Additionally, HL617 cells expressed AFP and connexin 43, suggesting that this cell line also contains cells with a more immature phenotype such as oval cells. In addition to RT-PCR analysis, these cell lines were stained with the A6 antibody, which recognizes both oval and biliary epithelial cells. In the HL617 population, this staining was less intense than that observed on the other cell lines. In addition, there were a number of cells that were not recognized by the A6 antibody. Immunohistochemistry using the A6 antibody on the BroC2 cells was performed while the cell line was being derived and was not studied in the established BroC2 cell line. Therefore, the reactivity of this cell line toward A6 early in culture and expression of hepatocellular markers at further passages may suggest that with continual passage the cell population became more hepatocellular in nature.

The differences in gene expression observed between the cell lines isolated from different genetic backgrounds is intriguing and may be due to different modifier genes present within the genetic backgrounds. The derivation of hepatocyte cell lines has been shown to be more likely to occur from mice with genetic backgrounds that predispose them to hepatocellular carcinomas, such as the C3H.[21] In addition, the liver lesion observed in *TgN737Rpw* mutant mice on the two genetic backgrounds differs, with a more aggressive cellular proliferation observed on the FVB/N background, whereas on the C3H genetic background, the lesion is more focal and shows an increase in portal fibrosis. Whether these differences in the mutant phenotype contributed to the cell type that was isolated is unclear.

In addition to the characterization of gene expression in these cell lines, experiments aimed at determining the growth characteristics of these cells *in vivo* were initiated. BroC2 cell clones were injected into the interscapular fat pad of isogenic mice, where they formed well defined ductular structures, suggesting that these cells are capable of differentiating down the biliary pathway. These data indicated that cells that expressed hepatocyte markers are capable of forming biliary structures when placed in the proper environment, suggesting that the BroC2 cells are capable of differentiating down bipotential pathways as has been described for oval cells.

The characterization of these cell lines revealed subtle differences between those derived from a mutant liver (BroF2) and from normal and heterozygous livers (LA and L2). Mainly, the mutant-derived BroF2 cell line did not express many of the genes associated with hepatocellular differentiation, whereas the LA and L2 cell lines did. Interestingly, the liver pathology observed in *TgN737Rpw* mice, which includes the proliferation of oval cells and ductular hyperplasia and dysplasia, might indicate that these cells in mutant animals are capable of differentiating down the biliary but not the hepatic pathway. Such a scenario would place *Tg737* at a pivotal position in the differentiation of this cell type.

Finally, the isolation of cell lines from mutant *TgN737Rpw* livers provides a valuable reagent in which we can directly test the function of *Tg737*. Using the HL617 mutant cell line, we initiated studies to determine the involvement of *Tg737* in cellular proliferation. Data from these experiments indicate that *Tg737* slows cellular proliferation. Whether *Tg737* exerts this effect by directly acting in the cell cycle or by altering the differentiation status and/or growth factor responsiveness in these cells is currently being tested.

In addition, these cells should prove useful in understanding the molecular events that lead to the liver pathology observed in *TgN737Rpw* mice. Given the similarities between *TgN737Rpw* mutant mice and ARPKD patients, such information may prove valuable in understanding the defects leading to ARPKD and should also prove useful in developing our understanding of molecular events leading to oval cell proliferation in pathological conditions, such as during hepatocarcinogenesis.

Acknowledgments

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