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BASIC STUDIES

Effect of mutant β-catenin on liver growth homeostasis and hepatocarcinogenesis in transgenic mice

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Abstract

Background: Mutations in the Wnt signalling pathway molecule β-catenin are associated with liver cancer. *Aims:* Our aim was to confirm the effects of stabilized β-catenin on liver growth, identify whether those effects were reversible and cell autonomous or non-cell autonomous and to model β-catenin-induced liver cancer in mice. *Methods:* Using a liver-specific inducible promoter, we generated transgenic mice in which the expression of mutant β-catenin can be induced or repressed within hepatocytes in mice of different ages. *Results:* Similar to other models, the hepatic expression of mutant β-catenin in our model beginning *in utero* or induced in quiescent adult liver resulted in a two-fold liver enlargement and development of disease with a latency of 1–5 months, and mice displayed elevated blood ammonia and altered hepatic gene expression. Our model additionally allowed us to discover that molecular and phenotypic abnormalities were reversible following the inhibition of transgene expression. Hepatocyte transplant studies indicated that mutant β-catenin could not increase the growth of transgene-expressing foci in either growth-permissive or -restrictive hepatic environments, but still directly altered hepatocyte gene expression. Mice with continuous but focal transgene expression developed hepatic neoplasms after the age of 1 year. *Conclusions:* Our findings indicate that hepatocyte gene expression is directly affected by mutant β-catenin in a cell autonomous manner. However, hepatomegaly associated with diffuse hepatocyte-specific expression of mutant β-catenin is secondary to liver functional alteration or non-cell autonomous. Both phenotypes are reversible. Nevertheless, some foci of transgene-expressing cells progressed to carcinoma, confirming the association of mutant β-catenin with liver cancer.

Protein-stabilizing point mutations or deletions of β-catenin, an intracellular messenger in the Wnt signalling pathway, have been reported in up to 50% of human and mouse hepatocellular carcinomas (HCC) (1–10). Several investigators have used transgenic mouse models to confirm the association of β-catenin mutations with liver growth abnormalities. Diffuse hepatocyte-directed expression of mutant β-catenin initiated in fetal liver causes hepatomegaly and loss of animal condition within 5 weeks after birth (11, 12). Diffuse expression initiated in adult liver causes hepatomegaly within 3 weeks of onset (12). Focal expression initiated in adults has no apparent effect on liver growth, and is not associated with altered hepatocyte foci or HCC (12). Mutant β-catenin only promotes cancer when combined with other carcinogenic alterations, including the focal expression of mutant Hras (13), loss of the serine/threonine kinase Lkb1 (14) or administration of diethylnitrosamine (15).

β-Catenin also plays a role in the metabolic zonation of the liver (16, 17). Several enzymes are expressed exclusively in perivenous hepatocytes. Membranous immunohistochemical staining of wild-type β-catenin protein in hepatocytes is most intense in the perivenous region (17). The expression of the adenomatous polyposis coli protein, a negative regulator of β-catenin, is strongest in periportal hepatocytes, where no β-catenin staining is noted. Benhamouche and colleagues inactivated adenomatous polyposis coli throughout mouse liver. Both β-catenin and perivenous-restricted genes were now expressed diffusely in the liver, indicating a loss of normal hepatic metabolic zonation (17). Furthermore, two-thirds of these mice also developed HCC (16).

This report describes a novel transgenic mouse model that extends our understanding of the effects of oncogenic β-catenin on liver growth homeostasis, including cancer. Our model provides the ability to regulate

organ-specific transgene expression in a temporal fashion, and allows us to quantify the influence of β-catenin on hepatocyte growth *in vivo*. We first describe model validation. We then use this model to address several questions that previous studies could not answer: (i) are β-catenin-induced lesions reversible? (ii) Are the effects of β-catenin cell autonomous or non-cell autonomous? And (iii) can we establish a model in which mutant β-catenin by itself induces mouse hepatic neoplasms *in vivo*?

Materials and methods

Experimental animals

Mice were housed and maintained according to The Guide for the Care and Use of Laboratory Animals in an AAALAC-accredited facility. Experimental procedures were approved by the Animal Care and Use Committee. Transgenic lines have been assigned the following genetic designations: MUP-uPA line 350-2, TgN (Mup-Plau)1Eps; hsMT-LacZ line 379-4, TgN (MtnlLacZ)4Eps; R26-hPAP line 808-6, TgN(R26ALPP)5Eps; tetOCMV-βcat line 1768-6, TgN(tetopCatnb$^{\Delta 89N}$)30Eps; tetOCMV-βcat line 1768-8, TgN(tetopCatnb$^{\Delta 89N}$)31Eps; and LAP-tTA, TgN(tTALap)5Uh.

Major urinary protein urokinase-type plasminogen activator (MUP-uPA), metallothionein-nLacZ (18), encoding a nuclear-localized β-galactosidase enzyme, and ROSA26 (R26)-human placental alkaline phosphatase (hPAP) transgenic mice were generated and identified as described (19–21). The liver-enriched activator protein tetracycline transactivator (LAP-tTA) transgene targets the expression of the tetracycline transactivator protein to hepatocytes, and has been described (22). The β-catenin cDNA construct harbouring a truncation of the amino-terminal 89 amino acids was provided by Dr Caroline Alexander at the University of Wisconsin-Madison and has been shown to be biologically active in mouse mammary epithelium (23). Oncogenic point mutations and deletions prevent β-catenin from being targeted for ubiquitin-mediated proteosomal degradation, thereby stabilizing the protein. Most transgenic mouse studies have used deletion mutants. Furthermore, Nejak-Bowen *et al.* (15) have shown that an activating point mutation did not provide TOPflash activity, a marker of β-catenin activity, in cultured cells. Therefore, we selected this mutant to ensure the activation of the Wnt signalling pathway in mouse hepatocytes. This mutant was also engineered to contain the myc-epitope-tag, a target for immunohistochemical detection. The construct was inserted downstream of a tet-operon-cytomegalovirus (tetOCMV) promoter (24). Two lines of tetOCMV-βcat-bearing transgenic mice were examined in this study, designated 1768-6 and 1768-8. tetOCMV-βcat mice were identified by polymerase chain reaction (PCR) of tail DNA using the tetO-1 forward primer, 5′-GAAAGTCGAGCTCGGTACCC-3′, and the reverse primer, 5′-GGCTCCATGGTGATACAAGGG-3′. Thermocycler conditions were as follows (1): 92 °C for 110 s (22); 35 cycles of 45 s at 92 °C, 60 s at 51 °C and 60 s at 72 °C; and (23) 72 °C for 5 min. MUP-uPA hepatocyte transplant recipient mice were in the FVB6(F1) strain background; all others were FVB. To inhibit the expression of the tetOCMV-βcat transgene, mice were administered doxycycline (Dox) in drinking water (5 mg/ml) or food (0.5 g/kg).

Comparative hepatocyte growth assay

Comparative hepatocyte growth assay (CHeGA) is a transplantation-based assay that we use to quantify the effect of oncogene expression on hepatocyte growth homeostasis *in vivo* (25). In this assay, two differentially marked populations of hepatocytes are isolated from two donor transgenic mice using a modified two-step EDTA/collagenase A protocol (21). One population expresses the LacZ-marker transgene. The second expresses the hPAP-marker transgene and mutant β-catenin. Hepatocytes are surgically transplanted via an intrasplenic injection into 3- to 4-week-old MUP-uPA-recipient mice within 6 h of isolation (21). The 10 μl cell suspension contains 3×10^4 hepatocytes from each donor population in a 1:1 ratio, for a total of 6×10^4 cells transplanted. At 3–4 weeks of age, recipient mice display uPA-mediated liver disease (21); transplanted hepatocytes travel from the spleen to the liver via the portal circulation, and approximately 7% engraft and clonally proliferate within diseased hepatic parenchyma as foci (26). By 4 weeks post-transplant, the hepatic parenchyma has been replaced by a mixture of foci of donor hepatocytes and endogenous healthy hepatocytes that no longer express the uPA transgene, and the liver becomes quiescent. The effect of growth-regulatory gene alterations on hepatocyte growth is evaluated by comparing the growth of the clonal progeny of the two types of donor hepatocytes within recipients at 2, 4, 8 and 12 weeks and > 1 year post-transplant using enzyme histochemistry to differentiate β-galactosidase from hPAP. The surface cross-sectional area of about 50 foci of the hPAP donor type, and at least 15 foci of the LacZ donor type, is determined for each recipient mouse. For each donor group evaluated, at least two separate donor cell preparations and multiple recipients are evaluated at each time-point post-transplant. Changes in hepatocyte growth characteristics due to altered growth-regulatory genes can be assessed in two distinct liver environments: a growth-permissive environment present up to 4 weeks post-transplantation and a growth-quiescent environment present from 4 to 12 weeks post-transplantation.

Detection and quantification of donor cell liver repopulation

β-Galactosidase- and hPAP-expressing hepatocyte foci were identified using histochemistry (21). Transgene expressing cells displayed a blue reaction product. The

cross-sectional surface area occupied by single blue-staining donor clones visible on the liver surface was determined under low-power magnification by measuring the major axes (a, b) of each stained focus, and then calculating the area of an oval using the formula $A = \pi[1/4(a+b)]^2$ (21).

Detection and quantification of hepatocyte apoptosis

Standard cytomorphological criteria associated with apoptosis including cell shrinkage and nuclear condensation and fragmentation were used to identify apoptotic hepatocytes. The hepatocyte apoptotic index was determined by expressing the total number of hepatocytes meeting the cytomorphological criteria of apoptosis as a percentage of the total number of hepatocytes counted (at least 1000) at $\times 200$ magnification. Global hepatic apoptotic activity was evaluated by quantifying gene expression of the pro-apoptotic protein Bax using quantitative PCR (QPCR).

Immunohistochemistry

One hour before euthanasia, all mice were administered 200 mg/kg bromodeoxyuridine (BrdU) intraperitoneally. BrdU is a nucleotide analogue incorporated into DNA of cells in the synthesis phase of cellular replication, and was detected immunohistochemically using an anti-BrdU rat monoclonal antibody (Accurate Scientific, Westbury, NY, USA). The BrdU labelling index was determined by expressing the number of BrdU-positive hepatocyte nuclei as a percentage of the total number of hepatocyte nuclei counted. An antibody against the myc-epitope-tag was used to detect the expression of the amino-terminally truncated β-catenin transgene in hepatocytes (Santa Cruz Biotechnology; sc-40, Santa Cruz, CA, USA). We used a mouse monoclonal antibody to detect glutamine synthase (BD Transduction Laboratories; Catalog no. #610518, Sparks, MD, USA).

Classification of lesions

Gross and microscopic examination of haematoxylin and eosin (H&E)-stained tissue sections was used to classify lesions in transplant recipient mice > 1 year of age. Altered hepatocyte foci were identified as isolated areas of parenchyma with a uniformly altered hepatocyte morphology, generally decreased hepatocyte size and increased basophilia, which compressed the surrounding normal-appearing parenchyma. Hepatocellular adenomas were identified as grossly visible masses composed of uniform-appearing hepatocytes but lacking in bile duct epithelial cells. Trabecular HCCs were identified as grossly visible masses in which hepatocytes were organized in cords that were greater than three cells in thickness.

Evaluation of blood ammonia levels

Blood was collected into an EDTA-coated tube (Fisher Scientific, 02-669-38, Waltham, MA, USA), thoroughly mixed and placed on ice. Samples were assessed for blood ammonia levels within 1 h of collection on a Hitachi 912 Chemistry Analyzer (Roche Diagnostics, Madison, WI, USA).

Quantitative polymerase chain reaction evaluation of hepatic gene expression

Total RNA was isolated from approximately 30 mg of mouse liver using the RNEasy Kit (Qiagen, 74104, Valencia, CA, USA). cDNA synthesis was carried out on 1 μg of RNA using the Protoscript First Strand cDNA Synthesis Kit (NEB, E6500, Ipswich, MA, USA) and included the dT23VN primer. Primers to detect the expression of selected genes in the liver were ordered from Integrated DNA Technologies and efficiencies were determined by performing triplicate QPCR reactions on serial 1:2 dilutions of mouse cDNA. Reactions devoid of template were included to detect any spurious product. The fitness of cDNA templates was determined using the SPUD assay (27) to detect the presence of QPCR inhibitors, and the 3′:5′ GAPDH assay (28) to measure RNA integrity. Utilizing genorm software (http://medgen.ugent.be/~jvdesomp/genorm/) and its underlying methodology (29), GAPDH, HPRT1 and TBP were selected from a group of 10 commonly used housekeeping genes as the most appropriate for mouse liver. QPCR reactions were carried out in 25 μl volumes on 96-well plates using the Biorad iQ5 (Hercules, CA, USA). Reactions contained $1 \times$ SYBR Green with fluorescein (Abgene, 1219, Rockford, IL, USA), 10 ng template and 300 nM of forward and reverse primer. Cycling conditions were as follows: one cycle at 95 °C for 15 min; 45 cycles of 95 °C for 10 s; and 60 °C for 60 s. Melt curve analysis was performed by ramping 0.5 °C/30 s from 60 to 95 °C. Quantification by means of the ddCT method was carried out using IQ5 software.

Results

Expression of mutant β-catenin beginning during fetal development

To generate transgenic mice with liver-specific expression of mutant β-catenin that initiated *in utero*, matings were set up between LAP-tTA transgenic and tetOCMV-βcat transgenic mice. The expression of the mutant β-catenin utilizing the tet-operon system requires the tetracycline transactivating protein (tTA) to bind to the tet-operon sequence and recruit transcriptional cofactors to initiate the transcription of the adjacent β-catenin coding region. Doxycycline (Dox, a tetracycline derivative) binds to the tTA protein, thereby preventing tTA from binding to the tet operon. In this system, administration of Dox to mice represses transgene expression. The removal of doxycycline initiates hepatocyte-specific expression of mutant

Table 1. Effect of hepatocyte-specific expression of mutant β-catenin on liver growth†

Dox status	No Dox (β-cat expressed *in utero*)			Off Dox at 6 weeks of age		
Transgenes, line	Control	tTA/β-cat 1768-6	tTA/β-cat 1768-8	Control	tTA/β-cat 1768-6	tTA/β-cat 1768-8
Age at analysis, weeks	5.3 ± 2.3 (31)	6.1 ± 2.7 (18)	4.0 ± 1.1 (15)	15.3 ± 6.1 (11)	27.3 ± 6.6 (5)	11.3 ± 1.0 (9)
Liver/body weight, %	6.9 ± 0.6 (31)	12.4 ± 1.8 (18)***	12.1 ± 2.4 (15)***	5.4 ± 0.7 (11)	8.2 ± 2.7 (5)**	10.2 ± 2.2 (9)***
BrdU index, %	0.7 ± 0.7 (16)	1.2 ± 0.5 (8)	0.7 ± 0.8 (8)	0.14 ± 0.13 (11)	1.5 ± 0.2 (5)*	1.0 ± 0.7 (5)**

†Data presented as mean ± SD (*n*). Control mice carried only single transgenes, and did not express β-catenin.
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$ vs. controls using the Mann–Whitney one-tailed test.
BrdU, bromodeoxyuridine; Dox, doxycycline.

β-catenin. No breeding pairs in this portion of the study received Dox, which allowed β-catenin expression in fetuses and offspring harbouring both transgenes.

Bitransgenic mice from each of two lines examined developed two-fold hepatomegaly and displayed signs of disease at a mean of 6.1 and 4.0 weeks of age respectively (Table 1). Livers from bitransgenic animals displayed no gross evidence of nodule formation, only diffuse liver enlargement. Microscopically, the livers appeared relatively normal (Fig. 1A and B). Increased BrdU labelling was not detected in these mice compared with the relatively high labelling index in 5-week-old control livers. Cytoplasmic and occasionally nuclear hepatocyte-specific expression of the mutant β-catenin construct were confirmed by immunohistochemistry (Fig. 1C). Glutamine synthase protein, a β-catenin target gene, was found in the liver from both bitransgenic and control mice. Control animals displayed intense cytoplasmic staining confined to hepatocytes immediately surrounding the central veins (as in Fig. 1H), whereas less intense glutamine synthase staining was present diffusely in bitransgenic mouse hepatocytes (Fig. 1F).

Expression of mutant β-catenin initiated in the adult liver

For this study, all breeding pairs were administered Dox to inhibit transgene expression in bitransgenic fetuses and offspring. Offspring remained on Dox until 6 weeks of age, at which time Dox was removed, initiating transgene expression in the hepatocytes of bitransgenic mice. Again, β-catenin expression resulted in two-fold hepatic enlargement, and latency differed according to the line (Table 1). Grossly, there was no evidence of nodule formation, only diffuse liver enlargement. The diffuse hepatomegaly in bitransgenic mice was accompanied by significant 7–10-fold increases in the BrdU labelling index compared with hepatocytes from age-matched control mice (Table 1). For line 1768-6, there was a significant increase in the apoptosis index of hepatocytes in bitransgenic mice compared with age-matched control mice using cytomorphologic criteria (Table 2). Proapoptotic Bax gene expression was also increased in line 1768-8 mice compared with age-matched controls (Table 2). The microscopic appearance of liver sections from both lines of bitransgenic mice was normal. Liver-specific expression of the transgene was confirmed using immunohistochemical detection of the myc-epitope-tag on six mice from each line (Fig. 1C). Glutamine synthetase was also localized. The results were the same as those for mice with fetal-induced expression (Fig. 1F).

Blood ammonia levels were assessed in mice with hepatomegaly and age-matched control mice. The mean blood ammonia level in mice expressing mutant β-catenin in hepatocytes was elevated over six-fold compared with the control mice (250 ± 160 vs. 39 ± 10 μmol/l respectively; $P = 0.004$).

Silencing expression of mutant β-catenin in adult liver

The results of the experiments reported above confirmed that our model reproduces the phenotypes reported for other models. However, those models could not identify the dependence of liver phenotype on continued β-catenin expression. To address this, we used mice from line 1768-8. Breeding pairs were administered Dox to inhibit transgene expression in bitransgenic fetuses and offspring. Offspring remained on Dox until 6 weeks of age, and then Dox was removed, initiating transgene expression in hepatocytes of bitransgenic mice. Bitransgenic mice were placed back on Dox to inhibit transgene expression at 5 weeks post-Dox removal (Table 2). Liver to body weight ratios rapidly reverted to normal, indicating that hepatomegaly requires continued transgene expression. Loss of liver-specific transgene expression was confirmed by QPCR. We were also unable to detect transgenic β-catenin on liver sections from bitransgenic mice by immunohistochemistry after Dox replacement (Fig. 1D and E). The usual zonal-restricted expression pattern of glutamine synthase had returned by 4 weeks post-Dox replacement (Fig. 1H). Cytomorphological criteria identified significant increases in the hepatocyte apoptotic index from mice expressing β-catenin to mice with transgene expression silenced for 5 days (Table 2), and the apoptosis index returned to normal by 28 days after transgene silencing. Furthermore, Bax expression was increased in mice expressing β-catenin compared with non-transgenic controls, and there was an additional increase in Bax expression by 5 days post-Dox replacement compared with all the other groups. The BrdU index was increased in mice expressing the transgene, but returned to normal by 5 days post-Dox

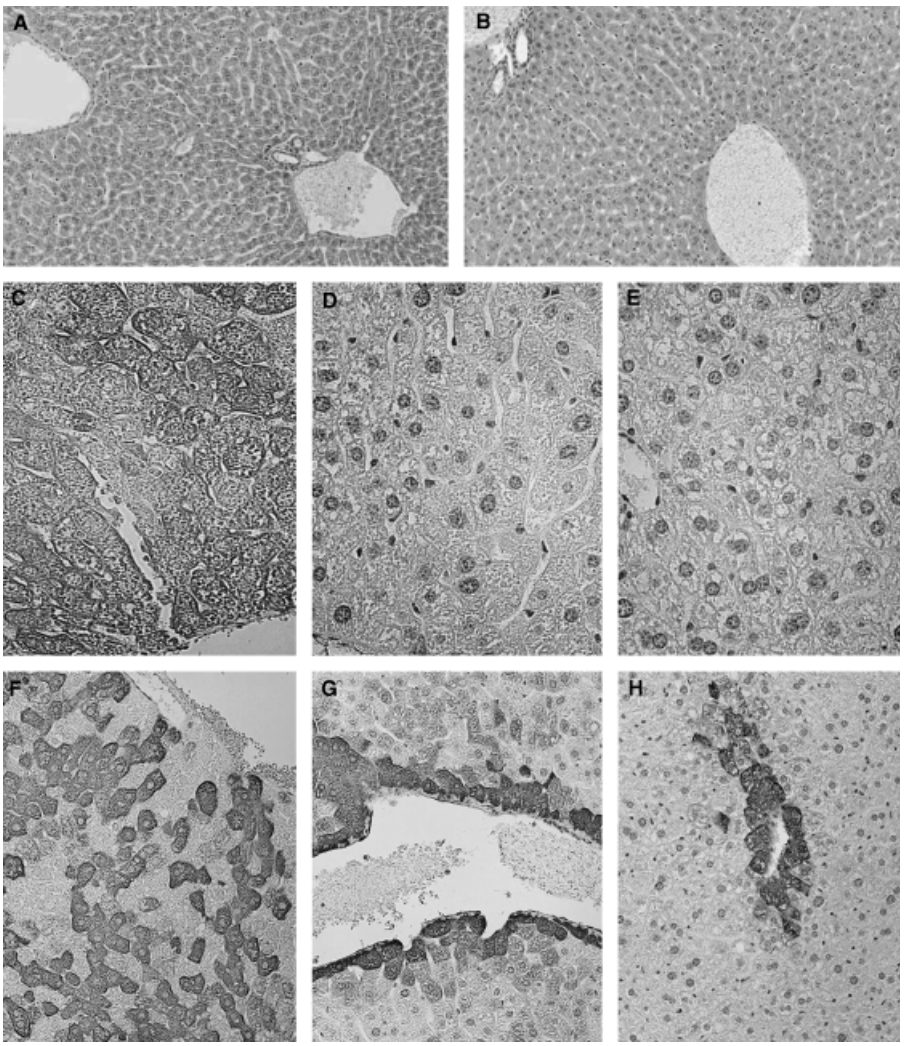

Fig. 1. Representative end-stage liver of age-matched non-transgenic control mice (A) and mice with hepatocyte-specific expression of mutant β-catenin initiated *in utero* (B). [(A), (B) haematoxylin and eosin). Transgene expressing liver resembles normal liver. Representative liver of end-stage mice with hepatocyte-specific expression of mutant β-catenin initiated at 6 weeks of age (C and F), and of similar mice after 10 days (D and G) and 4 weeks (E and H) of transgene suppression by Dox. (C, D and E) Anti-myc-tag (β-catenin) immunohistochemistry; (F, G and H) antiglutamine synthase immunohistochemistry. β-Catenin is expressed diffusely in tTA/β-cat bitransgenic liver, but expression is extinguished rapidly by administration of Dox. GS is also diffusely expressed in transgene-expressing liver. Following Dox administration, appropriate pericentral expression is re-established slowly, and is complete within 4 weeks. (A and B) × 100; (C–E) × 400; (F–H) × 200.

administration. Our findings support apoptosis as the cause of liver mass reduction.

To examine the scope of liver gene expression changes induced by β-catenin, we used QPCR to evaluate hepatic expression of 15 genes associated with nitrogen metabolism, carbohydrate or lipid metabolism, and cellular differentiation/proliferation (Table 3). β-Catenin altered the expression of genes in all categories. A subset of these genes was shown to be similarly differentially expressed in other models. Interestingly, the β-catenin target gene cyclin D1 was not changed significantly, consistent with two other reports (11, 15). The increased expression of Afp and decreased expression of Cyp2e1 suggest a mild reduction in hepatocyte differentiation. One month after Dox re-administration, which silenced β-catenin expression, most genes had returned to a normal level of expression, although two genes appeared to have overcompensated and were overexpressed.

The effect of a mutant β-catenin on transplanted hepatocytes

One previous study likely produced focal expression of activated mutant β-catenin in isolated hepatocytes (12).

Table 2. Reversibility of β-catenin-induced liver phenotype in line 1768-8†

Genotype (*n*)	Control (6)	tTA/β-cat (5)	tTA/β-cat (6)	tTA/β-cat (6)	tTA/β-cat (5)
Dox status	–	No Dox	5 day Dox	10 day Dox	28 day Dox
Transgene status	–	On	5 days off	10 days off	28 days off
Liver/body weight, %	–	13.1 ± 1.9‡	8.6 ± 0.9*‡	5.9 ± 0.8*	4.8 ± 0.7*
BrdU index, %	–	1.03 ± 0.68‡	0.17 ± 0.12*	0.00 ± 0.00*	0.00 ± 0.00*
Apoptosis index, %	0.03 ± 0.04	0.80 ± 0.09*#	3.4 ± 0.6*#	0.41 ± 0.18*#	0.10 ± 0.09*
Bax expression	1.00 ± 0.22	3.14 ± 0.76#	4.72 ± 0.72*#	1.34 ± 0.08*	1.53 ± 0.14*
β-cat transgene	–	1.0 ± 0.91	0.09 ± 0.02*	0.04 ± 0.01*	0.04 ± 0.01*

†Data presented as mean ± SD.

‡$P < 0.05$ vs. adult (15 week) control mice in Table 1 using the Mann–Whitney one-tailed test.

*$P < 0.05$ vs. no Dox samples using the Mann–Whitney two-tailed test.

# $P < 0.05$ vs. control samples using the Mann–Whitney two-tailed test.

BrdU, bromodeoxyuridine; Dox, doxycycline.

Table 3. Relative hepatic gene expression†

Genotype (*n*)	Non-transgenic (6)	Bitransgenic (5)	Bitransgenic (5)
Dox status	No Dox	No Dox	28 day Dox
β-Catenin transgene status	–	On	28 day off
Nitrogen metabolism			
Glutamine synthase (Gs)	1.00 ± 0.08	4.51 ± 4.29*	0.95 ± 0.22
Transporter of glutamate (Glt 1)	1.00 ± 0.22	2.64 ± 0.77*	0.63 ± 0.12
Ornithine aminotransferase (Oat)	1.00 ± 0.04	3.82 ± 1.83*	1.08 ± 0.14
Argininosuccinate lyase (Asl)	1.00 ± 0.05	3.86 ± 1.82*	1.71 ± 0.49*
Carbomoylphosphatase synthetase I (Cps)	1.00 ± 0.12	1.73 ± 0.70	1.60 ± 0.41*
Arginase (Arg 1)	1.00 ± 0.49	0.28 ± 0.14*	1.54 ± 0.12
Carbohydrate and lipid metabolism			
Glucose-6-phosphatase (G6p)	1.00 ± 0.49	0.90 ± 0.59	4.00 ± 1.65*
Ketohexokinase (Khk)	1.00 ± 0.19	0.47 ± 0.26*	1.97 ± 0.56*
Acetyl CoA carboxylase α (Acaca)	1.00 ± 0.15	1.92 ± 0.76 ($P = 0.10$)	1.31 ± 0.35
Cellular differentiation/proliferation			
Albumin (Alb)	1.00 ± 0.12	1.18 ± 1.02	1.12 ± 0.46
Alphafetoprotein (Afp)	1.00 ± 0.37	2.51 ± 1.07 ($P = 0.10$)	1.09 ± 0.41
Cytochrome p450 2e1 (Cyp2e1)	1.00 ± 0.13	0.65 ± 0.12*	0.98 ± 0.14
Cytochrome p450 7a1 (Cyp7a1)	1.00 ± 0.52	0.81 ± 0.24	0.82 ± 0.32
Y-box binding protein-1 (Yb-1)	1.00 ± 0.27	0.61 ± 0.05*	0.86 ± 0.10
Cyclin D1	1.00 ± 0.47	1.36 ± 0.51	1.60 ± 1.05

†Data expressed as mean ± SD.

*$P = 0.025$ vs. non-transgenic samples using the Mann–Whitney two-tailed test.

Dox, doxycycline.

However, the behaviour of β-catenin-expressing cells could not be assessed at the cellular level. Earlier studies also could not quantify the growth effects of β-catenin on hepatocytes in growth-permissive and -restrictive hepatic environments. To address these limitations, we used the CHeGA (described in 'Materials and methods') to measure the effect of mutant β-catenin on transplanted hepatocyte growth. Consistent with previous studies using CHeGA, there was no difference in the mean cross-sectional surface area between hPAP-only and LacZ-only hepatocyte populations at any time post-transplant, indicating that there was no differential effect on hepatocyte growth because of the marker-transgenes (21). Because variation in focus growth size existed among recipients at any time-point, we normalized the data by dividing the median focus area of hPAP-expressing foci by the median focus area of the LacZ foci in each mouse. The average of ratios calculated from all recipients at a specific time-point resulted in a focus ratio distribution (Table 4).

The effect of mutant β-catenin expression on transplanted hepatocyte growth is shown in Table 4. There was no significant increase in transplant focus growth during the growth-permissive phase, and no focus growth in quiescent liver. Immunohistochemically detectable transgenic β-catenin and glutamine synthase were identified in foci expressing hPAP in recipient livers, confirming transgene expression (Fig. 2A and B). The Tukey method

Table 4. Transplanted hepatocyte growth characteristics for line 1768-8

	Focus ratio distribution, mean ± SEM (*n*) Time post-transplant				Extreme outliers (%)
Transgene(s)	2 weeks	4 weeks	8 weeks	12 weeks	Mean ± SD (*n*)
hPAP only	1.1 ± 0.2 (8)	0.97 ± 0.2 (8)	0.97 ± 0.1 (8)	0.96 ± 0.1 (8)	1.5 ± 2 (8)
β-Cat 1768-6	1.0 ± 0.2 (9)	0.86 ± 0.1 (7)	0.86 ± 0.2 (8)	0.96 ± 0.3 (10)	1.8 ± 1.8 (10)
β-Cat 1768-8	1.2 ± 0.3 (7)	1.2 ± 0.3 (9)	1.0 ± 0.4 (12)	1.1 ± 0.3 (11)	1.1 ± 1.9 (11)

hPAP, human placental alkaline phosphatase.

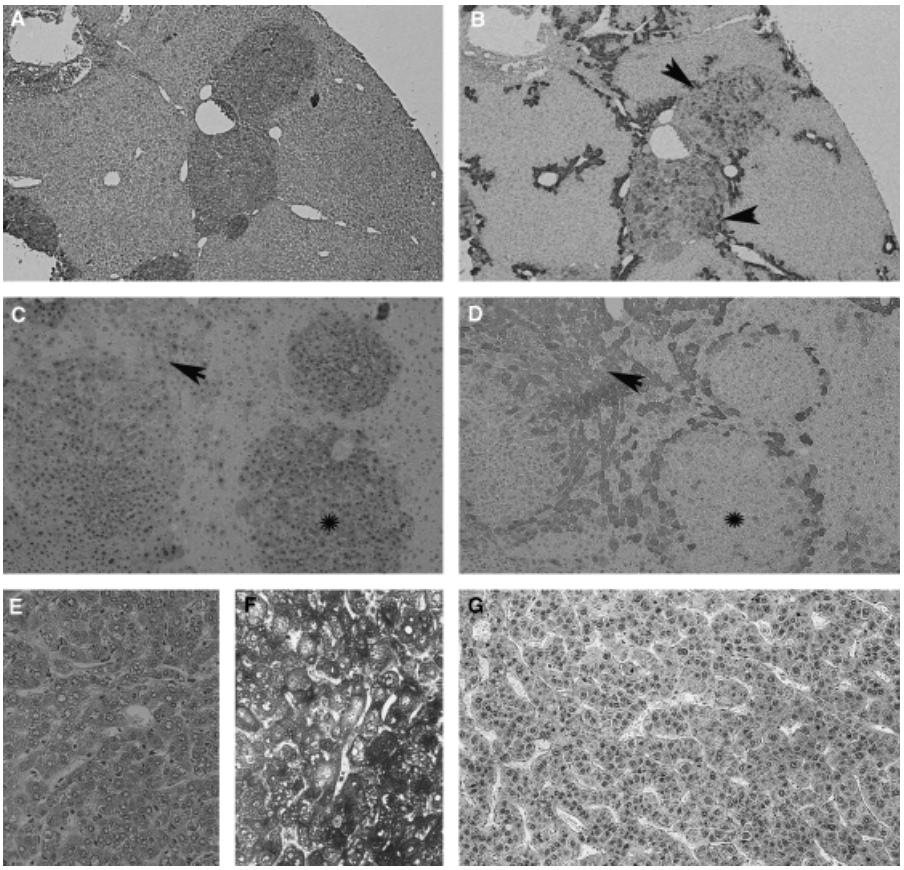

Fig. 2. (A and B) Representative liver of transplant recipient mice receiving β-catenin-expressing hepatocytes at 8 weeks post-transplantation. (A) Anti-myc-tag (β-catenin) immunohistochemistry; (B) antiglutamine synthase immunohistochemistry. Donor cell foci (arrows in B) are positive for both β-catenin and glutamine synthase. (C and D) Representative end-stage transplant recipient liver sections, showing altered hepatocyte foci (asterisk). (C) Anti-myc-tag (β-catenin) immunohistochemistry; and (D) antiglutamine synthase immunohistochemistry. Note that β-catenin-expressing cells within altered hepatocyte foci no longer express glutamine synthase, whereas β-catenin-expressing cells that are not part of an AHF retain glutamine synthase expression (arrow). (E–G) β-Catenin-induced adenoma [(E), haematoxylin and eosin (H&E); (F), hPAP histochemistry] and hepatocellular carcinomas [(G), H&E]. Lesions appear to be well differentiated. (A and B) × 100; (C–E) × 200.

was utilized to identify extreme outlier (EO) foci in each recipient liver (30). Foci in an individual recipient liver are considered EOs when their cross-sectional surface area is three times the interquartile range (distance between the first and the third quartiles) above the third quartile within their distribution. EOs from each animal at the 12-week time-point were averaged to give the EO for each donor hepatocyte genotype. EO foci have an extreme growth potential; in this regard, they display a preneoplastic phenotype. Hepatocyte foci did not show increased EO frequency at the 12-week time-point compared with control hepatocytes (Table 4).

A subset of transplant recipients was maintained until mice developed signs consistent with the development of disease (most notably loss of muscle mass in the caudal spine). Seven animals were collected at 55 ± 5 weeks post-transplantation. Six of the seven animals displayed enlarged livers with irregular liver margins and multifocal nodules. H&E-stained sections were made of representative liver lesions, and adjacent sections were stained for the expression of the hPAP-marker transgene, transgenic β-catenin and glutamine synthase. All six slides examined displayed at least one altered hepatocyte focus, four of the six had at least one hepatocellular adenoma and two had well-differentiated trabecular carcinomas (Fig. 2E and G). All lesions were derived from transplanted hepatocytes as demonstrated by hPAP staining (Fig. 2F). Interestingly, β-catenin-expressing cells in AHF lesions no longer expressed glutamine synthase (Fig. 2C and D). Donor-cell-derived lesions do not develop in mice that are recipients of marker-only hepatocytes. Therefore, focal mutant β-catenin expression in hepatocytes can induce hepatic neoplasia in this model, although with a long latency and low incidence; by itself, mutant β-catenin is weakly oncogenic.

Discussion

In this study, we define the effects of altered β-catenin expression on hepatocyte growth homeostasis in mice. Our system enables us to turn on and off the expression of mutant β-catenin in either fetal or adult liver. Using this single model, we found that deregulated hepatic expression of β-catenin is sufficient to induce rapid and diffuse liver enlargement regardless of the developmental stage of initiation, consistent with separate findings in other transgenic models (11, 12). Our model allowed us to demonstrate further that hepatomegaly is reversible when β-catenin expression is inhibited, via a mechanism consistent with apoptosis. This process is remarkably rapid, with most measures of liver size and cell turnover returning to normal by 10 days after transgene repression. Lesion reversibility suggests that mutant β-catenin-expressing cells are not characterized by high levels of genomic instability that could render those cells resistant to growth restraint following mutant β-catenin silencing.

Liver hyperplasia could be the result of a primary transgene effect or of compensatory growth of hepatocytes trying to replace a functional or a metabolic deficit induced by transgene expression. Information supporting the latter possibility for β-catenin comes from our finding of elevated blood ammonia and the identification of changes in the expression of several hepatic genes associated with ammonia metabolism, as reported before (11, 12, 17). Our data also suggest decreased carbohydrate and increased lipid metabolism, and a reduction in the hepatocyte differentiation state, although the expression of all genes was not affected. Importantly, our transplant studies demonstrated that hepatocyte foci expressing mutant β-catenin do not grow within an otherwise normal liver. If liver enlargement in mice diffusely expressing β-catenin in hepatocytes was caused by a primary transgene effect, then transplanted hepatocytes expressing mutant β-catenin would be expected to show an increase in hepatocyte focus growth in the transplantation assay. Thus, we have discovered that β-catenin growth effects are non-cell autonomous. In contrast, gene expression alterations induced by β-catenin are cell autonomous, as shown by maintenance of the altered pattern of glutamine synthase expression in β-catenin-expressing transplant foci.

The routine discovery of β-catenin mutations in human and mouse liver neoplasms suggests a causal role for β-catenin in this disease. In our study, transplant recipient mice developed well-differentiated hepatocellular neoplasms derived from foci of mutant β-catenin-expressing cells when over a year of age, demonstrating that the expression of mutant β-catenin in hepatocytes increases the risk of neoplasia. Mutant β-catenin was not oncogenic in other models, and could only promote carcinogenesis when combined with another oncogenic stimulus, including expression of mutant Hras (13), loss of Lkb1 (14) or administration of the carcinogen diethylnitrosamine (15). To begin to address the mechanism by which β-catenin contributes to hepatocarcinogenesis, we quantified the growth characteristics of β-catenin-expressing hepatocytes using CHeGA. Following transplantation into MUP-uPA mouse liver, β-catenin-expressing hepatocytes displayed no detectable alterations in hepatocyte growth either in growth-stimulatory or in quiescent hepatic environments, and did not increase the frequency of EO foci among transplanted cells. Unlike β-catenin, other oncogenic molecules examined using CHeGA produced distinct and quantifiable alterations in hepatocyte growth and/or increased the frequency of hepatocytes with extreme growth potential (25). Transforming growth factor (TGF)-α and c-myc increased the rate of hepatocyte growth in a growth-permissive environment, mutant Hras induced growth in a quiescent environment and simian virus 40 T-antigen, mutant Hras and the TGF-α/c-myc combination increased the frequency of EO foci. This marked difference in the behaviour of β-catenin vs. other oncogenes in the transplant assay provides additional support for the hypothesis that at least two pathways exist in the development of liver cancer, one associated with a high level of genomic instability and the second associated with normal chromosomal stability, a low-malignant phenotype and activation of Wnt signalling pathways (1, 2, 4, 31, 32). We conclude that the mechanisms by which β-catenin mutation contributes to hepatocarcinogenesis are distinct from those of other oncogenes, perhaps principally enhancing neoplastic progression of hepatocytes affected by other genetic alterations. We are testing this proposal by examining oncogene co-expression in our model.

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