Global Increase of p16\textsuperscript{INK4a} in APC-Deficient Mouse Liver Drives Clonal Growth of p16\textsuperscript{INK4a}-Negative Tumors

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Abstract

Reduction of b-catenin (CTNNB1) destroying complex components, for example, adenomatous polyposis coli (APC), induces b-catenin signaling and subsequently triggers activation of genes involved in proliferation and tumorigenesis. Though diminished expression of APC has organ-specific and threshold-dependent influence on the development of liver tumors in mice, the molecular basis is poorly understood. Therefore, a detailed investigation was conducted to determine the underlying mechanism in the development of liver tumors under reduced APC levels. Mouse liver at different developmental stages was analyzed in terms of b-catenin target genes including Cyp2c1, Glut, and Ihh using real-time RT-PCR, reporter gene assays, and immunohisto-logic methods with consideration of liver zonation. Data from human livers with mutations in APC derived from patients with familial adenomatous polyposis (FAP) were also included. Hepatocyte senescence was investigated by determining p16\textsuperscript{INK4a} expression level, presence of senescence-associated b-galactosidase activity, and assessing ploidy. A b-catenin activation of hepatocytes does not always result in b-catenin positive but unexpectedly also in mixed and b-catenin-negative tumors. In summary, a senescence-inducing program was found in hepatocytes with increased b-catenin levels and a positive selection of hepatocytes lacking p16\textsuperscript{INK4a}, by epigenetic silencing, drives the development of liver tumors in mice with reduced APC expression (APC\textsuperscript{-/} mice). The lack of p16\textsuperscript{INK4a} was also detected in liver tumors of mice with triggers other than APC reduction.

Implications: Epigenetic silencing of p16\textsuperscript{INK4a} in selected liver cells bypassing senescence is a general principle for development of liver tumors with b-catenin involvement in mice independent of the initial stimulus. Mol Cancer Res; 13(2); 239-49. ©2014 AACR.

Introduction

Genesis of hepatocellular carcinoma is not fully understood, though several carcinogenic pathways involved in this process were identified (1) among them the Wnt/b-catenin pathway. Activated b-catenin (CTNNB1) signaling contributes to approximately 30% of hepatocellular carcinomas (2,3) and is characterized by nuclear and/or cytoplasmic staining of b-catenin (4) which contrasts to cell membranous staining of unaffected liver. Consequently, Wnt/b-catenin target genes, i.e., glutamine synthetase (Glu; ref. 5), are upregulated in b-catenin–positive hepatocellular carcinomas. Induction of b-catenin signaling in hepatocellular carcinomas is caused by gain-of-function mutations in b-catenin (6) or mutations in genes coding for components of the b-catenin destruction complex, i.e., AXIN1 (7) or AXIN2 (8). Though mutations of adenomatous polyposis coli (APC) gene, a further constituent of b-catenin destruction complex, are rarely detected in primary liver cancer (9), methylation of APC promoter seems significant in hepatocellular carcinoma suggesting functional importance of altered APC levels (10). In contrast, other malignancies of gastrointestinal cancer, i.e., colon and rectal cancers, are strongly predisposed to APC germline mutations, as found in familial adenomatous polyposis (FAP; ref. 11). Recently, context-specific responsiveness for Wnt/b-catenin signaling has been suggested necessary to develop cancer following APC

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reduction (12). Buchert reported development of hepatocellular carcinomas in livers of aged homozygous Apc\(^{580S}\) mice expressing reduced APC levels (13). Unexpectedly, hepatocellular carcinomas of this model display a downregulation of the Wnt target gene Axin2 (14), suggesting its reduction in tumors might have promoted hepatocellular carcinoma formation at the long latencies observed in these mice.

However, the tumors lacked hypermethylation of Axin2 promoter (12). Moreover, additional events causing tumorigenesis in this model were still unclear as mutations in oncogenes, i.e., Hras, were not observed. Therefore, the mechanism of how APC reduction promotes hepatocellular carcinoma development remains ambiguous and important to elucidate.

Here, we investigate potential mechanisms supporting development of hepatocellular carcinomas in livers with reduced APC levels. To this end, we utilize Apc\(^{580S}\) mice (13) exhibiting elevated \(\beta\)-catenin levels and investigate deregulation of Wnt signaling during all stages of hepatocellular carcinoma development. By comparing expression pattern in isolated hepatocytes and liver tissue of transgenic mice with different levels of \(\beta\)-catenin, which were generated with the help of the tet-inducible expression system, we inferred a concerted action of a senescence-inducing program in hepatocytes with increased \(\beta\)-catenin levels and a positive selection for hepatocytes with loss of cell-cycle inhibitor p16\(^{INK4a}\) (CDKN2A; ref. 15) as driver for the development of liver tumors in Apc\(^{580S}\) mice.

**Materials and Methods**

**Mice**

An overview of transgenic mice is presented in Supplementary Table S1. Apc\(^{580S}\) mice (13), termed Apc\(^{580S}\), carry a homozygous floxed exon 14 Apc allele. Ctnnb1\(^{lox/lox}\) (16) mice, termed Ctnnb1\(^{lox/lox}\), were purchased from Jackson Laboratories. Three knockout (KO) mice, Apc\(^{IKO}\), Ctnnb1\(^{IKO}\), and Apc\(^{IKO}\)/Ctnnb1\(^{IKO}\) were obtained by interbreeding of floxed mice with liver-specific inducible Cre mice (17) carrying inducible P\(^{oTet}\)-Cre-recombinase (LC-1) and tetracycline controlled transactivator (TA\(^{LAP2}\)) transgenes (18). Homozygous P\(^{oTet}\)-Cre-Apc\(^{IKO}\) mice were bred with homozygous TA\(^{LAP2}\)/Apc\(^{IKO}\) mice to obtain P\(^{oTet}\)-Cre-TA\(^{LAP2}\)/Apc\(^{IKO}\), which after induction by doxycycline withdrawal result in Apc\(^{IKO}\) for breeding schema see Supplementary Fig. S1). Accordingly, interbreeding was performed with Ctnnb1\(^{lox/lox}\) and combined Apc\(^{IKO}/\)

**Histology and immunohistochemistry**

Immunohistochemistry was performed as described (19, 20). Briefly, 5 \(\mu\)m paraffin sections were dewaxed with xylol and hydrated through descending alcohol series. Antigen retrieval was assessed by microwaving in citrate buffer (pH 6.0). Slides were equilibrated in Tris-buffered saline (pH 7.4), quenched with H\(_2\)O\(_2\), blocked with biotin/avidin, and goat serum and blocking reagent (VECTOR M.O.M. Immunodetection Kit) for mouse antibodies, respectively, and incubated with primary antibodies (listed in Supplementary Table S2) overnight at 4°C. Corresponding biotinylated secondary antibodies were coated for 1 hour at room temperature. After washing slides were incubated with extravidin--POD conjugate and washed three times before staining with 3,3' diaminobenzidine-tetrahydrochloride (DAB). POD oxidizes DAB producing a brown precipitate. For p16\(^{INK4a}\) immunochemistry human liver sections were stained automatically by a Ventana Nexes autostainer (Ventana) using CINtec p16 (E6H4) immunohistochemistry.

**Senescence-associated \(\beta\)-galactosidase**

Frozen liver samples were cut and mounted on Superfrost plus slides (Menzel). Slides were dried 1 hour at room temperature and fixed with 0.5% glutaraldehyde in PBS 5 minutes at room temperature. After washing in PBS, slides were incubated in 40 mmol/L Na\(_2\)HPO\(_4\), 40 mmol/L citrate pH 6.0, stained overnight at 37°C with 1 mg/mL 5-bromo-4-iodo-3-indolyl-\(\beta\)-D-galactoside (X-Gal). POD oxidizes DAB producing a brown precipitate. For p16\(^{INK4a}\) immunohistochemistry human liver sections were stained automatically by a Ventana Nexes autostainer (Ventana) using CINtec p16 (E6H4) immunohistochemistry.

**Reporter enzyme**

Sections were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS pH 7.4 for 30 minutes at room temperature, washed three times for 15 minutes with 2 mmol/L MgCl\(_2\), 0.01% Nonidet P-40 in PBS and stained with 1 mg/mL X-Gal, 5 mmol/L K\(_3\)Fe(CN)\(_6\), 5 mmol/L K\(_4\)Fe(CN)\(_6\) for 36 to 40 hours at 37°C in the dark. Slides were counterstained with nuclear fast red.

**Isolation of hepatocytes, cell culture, and transfection**

Primary hepatocytes were isolated and cultivated as described (20) by collagenase perfusion (21). Peripheral and portal liver tissues were isolated by a modified digitonin/collagenase perfusion technique (22, 23) and transfected with TOP-Gau reporter plasmids (24) using Effectene (Qiagen). ApC silencing in vitro was achieved by transfection with ApC siRNA (APCMSS202103, APCMS202104, APCMSS202105, Invitrogen, 4 nmol/L) using Interferin (Peqlab).

**Gaussian luciferase assay**

Gaussian luciferase activity was measured in 10 \(\mu\)L supernatant of transfected hepatocytes with 50 \(\mu\)L assay reagent on Orion-II microplate luminometer (TiterTek-Berthold).

**RNA isolation and quantitative real-time reverse transcription PCR (qRT-PCR)**

Total RNA was isolated using PeqGOLD RNA Pure isolation system (PeqLab). RNA quality was assessed by gel electrophoresis and purity was estimated using the A\(_{260}/A_{280}\) ratio. Concentration was adjusted to 0.5 mg/mL. qRT-PCR was performed as
DNA from tumor and surrounding liver tissue was isolated using NucleoSpin Tissue Kit (Macherey & Nagel). DNA methylation was tested with the bisulfite conversion method using the EZ DNA-Methylation-Gold kit (Zymo Research) and p16<sup>INK4A</sup> promoter-specific primers (ref. 25; Supplementary Table S3).

**Clinical chemistry**

Alanine aminotransferase (ALT) and glutamate dehydrogenase (GLDH) were measured on an automated clinical chemistry analyzer (Modular PPE, Roche).

**Flow cytometry**

Hepatocytes were counted and 200,000 cells were resuspended in 100 μL wash buffer (PBS, 5% FCS). After washing, 500 μL prechilled ethanol (−20°C) was added slowly with continuous mixing avoiding agglutination. Hepatocytes were kept on ice, washed twice with 1 ml PBS/5% FCS, and incubated for 1 hour with RNase A (1 mg/mL PBS) at 37°C. Suspensions were brought up to 200 μL with PBS/5% FCS and 20 μl propidium iodide (1 mg/mL) was added. This hepatocyte suspension was measured on a flow cytometer (FACSscan, BD Biosciences) for determining the ploidy grade.

**Statistical analysis**

All data are expressed as mean ± SEM. Statistical analysis was performed by the Student t test or Mann–Whitney test using SigmaPlot 11 (SSP Science). The accepted level of significance was set at P < 0.05.
Results

Activation of β-catenin initiates AXIN2 expression in selected individual hepatocellular carcinomas of aged Apc<sup> homo </sup>mice

Using liver sections, β-catenin signaling was investigated by immunohistochemistry in hepatocytes of young (8 weeks) and aged (10 months) Apc<sup> homo </sup>mice and compared with Apc<sup>KO </sup>mice representing a positive control for β-catenin activation (Fig. 1A–D). This staining displayed only few hepatocytes with activated β-catenin (nuclear expression) both in liver tissue from young Apc<sup> homo </sup>mice and tissue adjacent to tumors in aged mice. Liver tumors and their prestats in aged Apc<sup> homo </sup>mice exhibit, unexpectedly, a highly heterogeneous β-catenin staining in the same animal. Both β-catenin–negative (Fig. 1C, C) and β-catenin–positive (Fig. 1D, D) tumors were detected. Moreover, if tumors contained activated β-catenin, they always displayed membrane-bound β-catenin providing a mixed phenotype, while an exclusively nuclear or cytoplasmic staining was never detectable.

Quantification of the universal and direct Wnt/β-catenin target gene, Axin2 mRNA level in liver tissue extracts (Fig. 1E), expectedly revealed a significant elevation in Apc<sup> homo </sup> and Apc<sup>KO </sup> and a decrease by trend in Ctnnb1<sup>KO </sup> mice (only 2 animals were investigated due to sudden early death of Ctnnb1<sup>KO </sup> mice) and a significant decrease in Apc<sup>KO </sup>/Ctnnb1<sup>KO </sup> mice compared with controls (both heterozygous and wild-type mice). In contrast, Axin2 mRNA levels in tumor extract (containing several tumors) were not significantly altered (Fig. 1E). However, re-evaluation of macropiscopically visible tumors and subsequent subsection of individual tumors of each liver revealed different individual Axin2 mRNA levels (Fig. 1F). Thereby, in the same animal either up- or downregulation of the β-catenin–responsive target Axin2 occur in individual hepatocellular carcinomas. To functionally validate activation of β-catenin signaling in vivo, we transfected a TCF reporter, which allows monitoring activity of β-catenin triggers, in isolated primary hepatocytes (Fig. 1G). Hepatocytes from Apc<sup> homo </sup> mice show a 5-fold induction of Gaussia luciferase activity compared with controls, and in turn represent about one—fifth of luciferase activity obtained in Apc<sup>KO </sup> mice (Fig. 1G), thus supporting Axin2 mRNA data.

In vivo, we also confirmed the elevated activation of β-catenin signaling in Apc<sup> homo </sup> mice utilizing the Wnt reporter mouse Conductin<sup>lacZ </sup> in which the reporter gene β-galactosidase is expressed in response to the endogenous Conductin (Axin2) promoter. In Apc<sup> homo </sup>/Conductin<sup>lacZ </sup> mice β-galactosidase was detected only in pericentral hepatocytes as a weak spot-like staining within the nucleus of the first row of cells around central veins and rarely in the second row, while midzonal or perportal hepatocytes were never positively marked (Fig. 1H). In Apc<sup> homo </sup> mice (Fig. 1I) the expression zone expanded and staining intensity increased. Here, several individual midzonal hepatocytes show β-galactosidase activity.

β-catenin target genes are heterogeneously expressed in tumors and lesions

We examined expression of common β-catenin target genes, Glu and Cyp2e1 (Cytochrome P450 2E1; Fig. 2A), representing classical pericentral (pc) expressed proteins within livers of Apc<sup> homo </sup> mice at different ages. In addition, we inspected the pattern of typical periportally (pp) expressed proteins, carbamoyl phosphate synthetase I (CPS) and E-cadherin, which are expected to be conversely localized to pc-specific proteins (Fig. 2A). Liver tissue of Apc<sup> homo </sup> mice showed age-dependent upregulation of β-catenin target genes, Cyp2e1 and Glu, supporting data from Buchet and colleagues (12), who investigated five-month-old mice. The cellular proportion of the pericentral expression type in the zonal expression pattern starts to increase at age 3 weeks and is nearly completed at age 8 weeks. Later on, in mice, over 5 months old (Fig. 2A), first cancerous lesions appear. Although there was a slight increase of hepatocytes exhibiting nuclear/cytoplasmic β-catenin in aged Apc<sup> homo </sup> mice, GLUll expression never spread over the whole lobulus.

Figure 2.

Immunohistochemistry of marker proteins zonally expressed in mouse and human liver. A, a spreading of pericentral (GLUL, CYP2E1) and periportal (CPS, E-cadherin)-specific proteins over the liver lobe in Apc<sup> homo </sup> mice at different ages. Using rabbit anti-CPS I, mouse anti-GLUL, mouse anti-E-cadherin, and rabbit anti-CYP2E1 antibodies the distribution of indicated pericentral and periporal proteins is immunohistochemically demonstrated (brown, DAB). Liver sections of 8-week-old Apc<sup> homo </sup> mice, used as controls, show the localization of the proteins in normal mouse liver, because no differences were detected between C57BL/6 and Apc<sup> homo </sup> mouse liver at different ages (not shown). Central veins were indicated by cv, portal tracts by pv. Bar represents 100 μm. B, distribution of the pericentral marker enzyme GLUL in human liver sections. Immunohistochemistry with anti-GLUL antibody (brown, DAB) was performed on representative normal human liver sections and liver sections of patients with FAP.

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The shifted zonal expression pattern of pericentral and periportal proteins in liver parenchyma of Apchomo mice as a result of a continuous moderate activation of β-catenin signaling raised the question whether zonal shifting in liver parenchyma also occurs in patients with APC-repressing mutations. Thus, we examined the zonal expression pattern of GLUL in paraffin sections of livers of patients with FAP (Fig. 2B). In six of seven FAP liver samples, enlargement of the GLUL-positive zone was detected. In two of these six samples, GLUL-positive focal nodular hyperplasia was demonstrated (Fig. 2B, top right), whereas GLUL-positive nodules were not present in controls.

Precancerous lesions and tumors of aged Apchomo mice, however, feature surprising heterogeneity regarding the expression of the β-catenin target genes, Glul and Cyp2e1 (Fig. 3). As expected from nuclear/cytoplasmic β-catenin staining pattern and Axin2 mRNA quantification tumors with pericentral, periportal, or mixed expression pattern were detected within an individual animal (Fig. 3).

The mechanism driving carcinogenesis in Apchomo mice seems obscure, because β-catenin–dependent hepatocyte proliferation is supposed not to be cell-autonomous (26) and tumors with homogeneously nuclear β-catenin staining were not found in our experiments. Moreover, hyperproliferation neither of hepatocytes carrying nuclear nor cytoplasmic β-catenin nor of pc-reprogrammed GLUL-positive hepatocytes occurs in Apchomo mice (Supplementary Fig. S2). A pronounced proliferation in nonumbrous liver parenchyma was detected in livers of ApcKO mice only (Supplementary Fig. S2C) compared with coeval Apchomo and control mice (Supplementary Fig. S2A and S2B). However, the hyperproliferation in ApcKO mice is accompanied with ruin of hepatocytes by necrosis (Fig. 7A and B; black arrows). However, preliminary microarray experiments comparing the mRNA expression pattern of pericentral hepatocytes from Apchomo mice to periportal hepatocytes from Apchomo mice indicate alterations of tumor suppressor levels in pericentral hepatocytes. Furthermore, tumor suppressor p16INK4a was recently identified as a β-catenin target gene (27). Both results suggest p16INK4a as a possible key regulator for tumorigenesis in Apchomo mice. Consequently, we analyzed p16INK4a immunohistochemically and detected a very high cytoplasmic amount of p16INK4a in nearly all hepatocytes in aged Apchomo mice independently of their acinar location (Supplementary Fig. S3 and S4). Livers of patients with FAP were also examined concerning expression of p16INK4a. Hereby, both stainings manually performed with monoclonal antibody D7D7 used for detection of p16INK4a-negative lesions in mice (not shown) and automatically performed with monoclonal antibody E6H4 (CINtec p16; Supplementary Fig. S3) detected a pronounced incidence of p16INK4a in all livers samples of patients with FAP compared with normal liver. Thereby, a preference of p16INK4a expression in pericentral areas was observed in both FAP livers and controls (frames in Supplementary Fig. S3J and S3K and the corresponding magnifications I and K).

Simultaneously, loss of p16INK4a protein in all mouse liver tumors and precancersous lesions (Fig. 4A) was detected. Serial sections of liver tissue from mice at age 10 months or older showing macroscopically visible tumors corroborate the p16INK4a negativity of tumors and prestages independently on whether a periporal or pericentral expression program was followed (Fig. 4A). Cells within the p16INK4a-negative tumors proliferate which is shown by BrdU incorporation (Supplementary Fig. S2E, E'). In contrast tumor, surrounding hepatocytes which
are characterized by high p16INK4a expression do not proliferate (Supplementary Fig. S2E).

To confirm p16INK4a protein deficiency as a general hallmark of hepatocellular carcinomas in mice with abnormal β-catenin signaling, we stained liver sections harboring tumors, in which initial causative event had been identified as activating mutations in exon 3 of the CTNNB1 proto-oncogene, leading to constitutively active Wnt/β-catenin signaling, (ref. 28; Fig. 4C) and tumors, which developed after transfection of Apc<sub>flox</sub> mice using a virally encoded Cre-recombinase (ref. 29; Fig. 4B). All tumors including those with different genesis were p16INK4a protein negative. In contrast, liver tumors of aged Apc<sup>homo</sup> mice displayed decreased p16INK4a mRNA compared to control (see Supplementary Fig. S5). qRT-PCR of Psme3 mRNA in liver extracts of indicated mice and tumors of Apc<sup>homo</sup> mice and in separately isolated individual tumors (see also legend for Fig. 1); *, P < 0.05, Mann-Whitney test.

Figure 5.

Cause of p16<sup>INK4a</sup> reduction in mouse liver tumors. A, methylation-specific PCR proved epigenetic silencing of p16<sup>INK4a</sup> promoter. DNA of seven different tumors was examined by PCR using primers specific for methylated (m) and unmethylated (um) p16<sup>INK4a</sup>-promoter. As controls, a methylated DNA (positive-m; Zymed research) and DNA of tumor T1-1 surrounding liver tissue of an Apc<sup>homo</sup> mouse (homo-m, homo-um) were used. Abbreviations: see legend of Fig. 1B.

B, quantification of p16<sup>INK4a</sup> expression in isolated hepatocytes and liver tissue extracts by qRT-PCR. Mean relative mRNA content of pericentral hepatocytes of Apc<sup>homo</sup> (pc-Apc<sup>homo</sup>, n = 6), hepatocytes of Apc<sup>homo</sup> (hep-Apc<sup>homo</sup>, n = 3), Apc<sup>homo</sup> liver tissue (tissue-Apc<sup>homo</sup>, n = 6), and tumor tissue of Apc<sup>homo</sup> mice (n = 7) was compared with the mean value provided by hepatocytes of 5 control animals which were used as reference. The highest, but equal, p16<sup>INK4a</sup> expression was measured in tumor and surrounding liver tissue of Apc<sup>homo</sup> mice. *, P < 0.05, Mann-Whitney test. Tissue of tumor 2–2 possesses decreased p16<sup>INK4a</sup> mRNA compared to control (see Supplementary Fig. S5). C, qRT-PCR of Psme3 mRNA in liver extracts of indicated mice and tumors of Apc<sup>homo</sup> mice and in separately isolated individual tumors (see also legend for Fig. 1); *, P < 0.05, Mann-Whitney test.

Figure 5.
Cause of p16\textsuperscript{INK4a} deficiency in tumors of Ap\textsuperscript{homo} mice

The downregulation of p16\textsuperscript{INK4a} protein in tumors of Ap\textsuperscript{homo} mice matches data on kidney (30), showing that only bypassing senescence caused by p21\textsuperscript{CIP1} triggers renal tumors in Ap\textsuperscript{homo} mice. In liver, p16\textsuperscript{INK4a} silencing seems to meet this function.

To validate whether p16\textsuperscript{INK4a} reduction is epigenetically regulated p16\textsuperscript{INK4a} promoter methylation was examined. Only 1 of 7 tumors displayed a methylation-specific PCR product (Fig. 5A). Next, we investigated the transcriptional level by quantifying p16\textsuperscript{INK4a} mRNA by qRT-PCR. No reduction of p16\textsuperscript{INK4a} mRNA was detected in tumors compared with surrounding normal liver tissue of Ap\textsuperscript{homo} mice (Fig. 5B, right). In contrast, paradoxically, an increase of p16\textsuperscript{INK4a} mRNA level was detected in all tumors lacking p16\textsuperscript{INK4a} promoter methylation (Fig. 5B and Supplementary Fig. S5). The p16\textsuperscript{INK4a} reduction seems posttranscriptionally regulated, probably by increased degradation of p16\textsuperscript{INK4a} protein which would explain increased p16\textsuperscript{INK4a} mRNA levels as compensatory mechanism. Recently, p16\textsuperscript{INK4a} degradation was suggested to occur ubiquitn independently by PSME3 proteasome (31). Hence, we quantified Psme3 in tumor and liver extracts and found an upregulation exclusively in tumor tissue (Fig. 5C). Tumor T2–2 in which the p16\textsuperscript{INK4a} promoter is methylated shows no upregulation of Psme3 but demonstrates alternatives for p16\textsuperscript{INK4a} reduction in certain cases. Accordingly, this tumor was excluded for statistical analysis of Psme3 mRNA.

Consequences of altered p16\textsuperscript{INK4a} expression

Overexpression of tumor suppressors, e.g. p16\textsuperscript{INK4a} leads to induction of SA-\beta-Gal (GLB1; refs. 32, 33), activation of the facultative stem cell compartment, oval cells, in phases with proliferation demand (33) and polyploidization of hepatocytes (33). In all stages of life, more SA-\beta-Gal was detected in cryosections of Ap\textsuperscript{homo} compared with Ap\textsuperscript{homo} mice (Fig. 6B). However, stronger upregulation of SA-\beta-Gal was found in livers of Ap\textsuperscript{KO} mice (Fig. 6C) and the strongest reactivity was observed in tissue surrounding tumors and precancerosus lesions (Fig. 6D). Isolated primary hepatocytes of aged mice revealed in FACS analysis an increase of >16N ploidy in Ap\textsuperscript{homo} and Ap\textsuperscript{homo} mice (Fig. 6E), whereas hepatocytes with 4N ploidy decreased significantly in Ap\textsuperscript{KO} mice and by trend in Ap\textsuperscript{homo} mice. Immunohistologic stainings with anti-pan-cytokeratin antibody specific for oval cells (34) show their activation in Ap\textsuperscript{homo} mice starting at age 5 months. In livers of mice older than 10 months, both around macroscopically identifiable tumors and areas of cancer prestages,
a border of oval cells was visible (Fig. 6J and K, arrows). Hence, the question arises what trigger, preferably produced by tumors, could activate oval cell compartment in Apchomo mice. Recently, Indian hedgehog (IHH) was confirmed as an activator of hepatic stem cells produced by dying hepatocytes (35). We found Ihh upregulated both in cultured hepatocytes after Apc siRNA treatment (Fig. 6F) and in ApcKO mice (Fig. 6F) thus confirming Ihh increase in livers of AlcCre-ApcKO mice (36). These data combined with a trend of Ihh reduction in hepatocytes of Ctnnb1KO mice confirm Ihh as direct target of β-catenin (37). The upregulation of Ihh mRNA level in tumors (Fig. 6F) supports a role as death signal of hepatocytes and cell damage should be expected.

Evidence of hepatocyte damage

Necrotic cell death occurs in ApcKO mice of every age but also in old Apchomo mice with tumors and prestages (Fig. 7A and B).
addition, apoptotic death featured by numerous Councilman bodies (Fig. 7C), seems to play a role in hepatocyte loss of ApcKO mice, at least in aged mice, even though caspase-3 detection failed in all liver slides (not shown). As elevated liver enzymes in serum clearly indicate necrotic loss of hepatocytes, we measured characteristic liver parameters in serum of Apcshomo, Apchetero, and ApcKO mice. Although a slight, nonsignificant raise of ALT and GLDH activities was detected age dependently in Apcshomo up to the age of 12 months (Fig. 7D and F), a significant difference of these enzyme activities as shown for old mice (Fig. 7B and D) between Apcshomo and Apchetero mice (controls) could be measured at all these ages (not shown). In contrast, serum levels of tumor harboring mice and ApcKO mice were increased up to 10-fold compared with age-related Apc+/+ mice (Fig. 7A–D).

In addition, DNA damage was observed by anti–γ-H2AX staining in all tumors and their prestages (Fig. 7K). Moreover, large quantities of γ-H2AX–positive nuclei were detected in ApcKO mice (Fig. 7J).

Discussion

Reduced APC expression leads to permanent activation of β-catenin and consequently, to upregulation of Wnt/β-catenin target genes and a pericentral expression program (12, 28, 38). Conversely, Hras mutations cause a periportal expression program (28). Unexpectedly, livers of Apcshomo mice, providing a model of half-maximal β-catenin activation, develop phenotypically different tumors. Some of them are distinguished by the expression of the Wnt target gene Glul and others by detection of E-cadherin, which is actually downregulated by sufficient β-catenin activation and belongs to the periportal expression program. Other Wnt target genes, i.e., Axin2, are reduced or unchanged in such tumors (ref. 12; current study). Likewise, the peripherally expressed protein CPS is heterogeneously expressed.

The tumor suppressor p16INK4a, recently identified as a β-catenin target gene (27), was completely lost at protein levels in all tumors and precancerous lesions investigated here and results in bypassing senescence as recently also found in human hepatocellular carcinoma and hepatoblastoma (39, 40).

Our data suggest the epigenetic silencing of p16INK4a in selected liver cells is a common factor for development of liver tumors. First, APC reduction leads to overexpression of p16INK4a in the unaffected healthy liver of Apcshomo mice and in hepatocyes of ApcKO mice with highly activated β-catenin signaling. Subsequently, elevated p16INK4a induces senescence and protects from tumorigenesis as recently shown by others for premalignant hepatocytes in mice (41). Necessarily, increased ALT and GLDH levels in Apcshomo mice indicate loss of pericentrally programmed hepatocytes, which culminates in permanent proliferative stress of residual hepatocytes, being still senescent by p16INK4a overexpression. Therefore, no fully replicative senescence seems to occur, because growth and physiologic function of liver of Apcshomo mice are not limited up to a critical age of about 10 months when tumors develop. Because the replicative capacity of hepatocytes is impaired by p16INK4a overexpression, a physiologically required hepatocyte replacement generates a selective pressure, both forcing the initiation of p16INK4a silencing in selected populations of hepatocytes and promoting subsequent proliferation of clusters of p16INK4a-negative hepatocytes. We hypothesize this sequence of events because no single p16INK4a-negative hepatocyte was detected in younger animals before precancerous lesions occur and hyperproliferation does also not occur by moderate/half-maximal activated hepatocytes.

It seems irrelevant which metabolic program (pericentral/periportal phenotype) is followed by p16INK4a–silenced cells. The most relevant process responsible for silencing of p16INK4a function is the specific p16INK4a removal, most likely by PSME3 mediated proteasomal digestion.

The p16INK4a overexpression induced by diminished APC levels supports faster ageing and death of hepatocytes, which therefore contain a diminished proliferative capacity. A similar explanation can be supposed to pericentral hepatocytes, which have a continuous β-catenin signaling (42), and might also be relevant in liver parenchyma of patients with FAP as shown here by GLUL expression. P16INK4a upregulation as a consequence of APC-repressing mutations also occurs in hepatocytes of patients with FAP and likely protects from liver cancer in early stages of life in the majority of cases. The high incidence of hepatoblastoma in children with FAP (43) and the frequent occurrence of p16INK4a loss in hepatoblastoma (40) underscores the significance of the tumor suppressor p16INK4a in β-catenin–activated liver parenchyma.

The escalation of ALT and GLDH concentrations in ApcKO mice indicates a massive decay of hepatocytes probably causing sudden death of ApcKO mice 10 to 14 days after conditional knockout. The strong γ-H2AX staining in ApcKO mice verifying the replicative stress in ApcKO hepatocytes supports this suggestion.

The IHH signal delivered by dying hepatocytes is obviously not sufficient to activate an adequate number of oval cells to replace lost cells. This also applies to tumors whose elevated Ihh levels might follow, additionally to activation by β-catenin, damage-induced signals, similarly as reported on radiated hepatocytes (44). Even if sufficient oval cells were activated, their subsequent differentiation into hepatocytes would ultimately end in their decline.

Summarizing, our data suggest the epigenetic silencing of p16INK4a in selected liver cells bypassing senescence is a common principle for the development of tumors in mouse liver with β-catenin involvement independent of the initial stimulus.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Global Increase of p16\textsuperscript{INK4a} in APC-Deficient Mouse Liver Drives Clonal Growth of p16\textsuperscript{INK4a}-Negative Tumors

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