\(\beta\)-catenin activation in a novel liver progenitor cell type is sufficient to cause hepatocellular carcinoma and hepatoblastoma

Sharada Mokkapati\(^1\), Katharina Niopek\(^1\), Le Huang\(^1,2\), Kegan J. Cunniff\(^1\), E. Cristy Ruteshouser\(^1\), Mark deCaestecker\(^4\), Milton J. Finegold\(^5\), Vicki Huff\(^1,2,3\)

\(^1\)Department of Genetics, University of Texas MD Anderson Cancer Center, Houston Texas, Graduate Program in \(^2\)Genes and Development and \(^3\)Human Molecular Genetics, UT-Houston Graduate School of Biomedical Sciences, Houston, Texas, \(^4\)Department of Medicine, Vanderbilt University, Nashville, Tennessee, \(^5\)Baylor College of Medicine and Texas Childrens Hospital, Houston, Texas

Contact Information

Dr. Vicki Huff
Department of Genetics
UT MD Anderson Cancer Center
1515 Holcombe Boulevard
Houston, Texas, 77030
TEL: 713 834-6384
Fax: 713 834-6380
Email: vhuff@mdanderson.org

Financial Support

Support from NIH grants CA34936, DK069599, NCI CCSG grant CA16672, CPRIT RP100329, and CPRIT RP110324. SM is a recipient of the Dodie P. Hawn Fellowship in Genetics at MD Anderson Cancer Center.

Conflict of interest

No conflicts to disclose.
Abstract

Hepatocellular carcinoma (HCC) was thought historically to arise from hepatocytes, but gene expression studies have suggested it can also arise from fetal progenitor cells or their adult progenitor progeny. Here we report the identification of a unique population of fetal liver progenitor cells in mice that can serve as a cell of origin in HCC development. In the transgenic model used, mice carry the Cited1-CreERTM-GFP BAC transgene in which a tamoxifen-inducible Cre (CreER TM) and GFP are controlled by a 190kb 5' genomic region of Cited1, a transcriptional co-activator protein for CBP/p300 implicated in Wilms' tumors where Wnt signaling is activated. Wnt signaling is critical for regulating self-renewal of progenitor/stem cells and has been implicated in the etiology of cancers of rapidly self-renewing tissues, so we hypothesized that Wnt pathway activation in CreERTM-GFP progenitors would result in HCC. In livers from the mouse model, transgene-expressing cells represented 4% of liver cells at E11.5 when other markers were expressed characteristic of the hepatic stem/progenitor cells that give rise to adult hepatocytes, cholangiocytes and SOX9+ periductal cells. By 26 weeks of age, >90% of Cited1-CreERTM-GFP; Ctnnb1ex3(fl) mice with Wnt pathway activation developed HCC and, in some cases, hepatoblastomas (HB) and lung metastases. HCC and HB resembled their human counterparts histologically, showing activation of Wnt, Ras/Raf/MAPK and PI3K/AKT/mTOR pathways, and expressing relevant stem/progenitor cell markers. Our results show that Wnt pathway activation is sufficient for malignant transformation of these unique liver progenitor cells, offering functional support for a fetal/adult progenitor origin of some human HCC. We believe this model may offer a valuable new tool to improve understanding of the cellular etiology and biology of HCC and HB and the development of improved therapeutics for these
diseases.
Introduction

Hepatocellular cancer (HCC) is the fifth most common cancer worldwide with a very high mortality rate (1). Historically, HCCs were thought to arise from hepatocytes. Interestingly, gene expression profiling of human HCCs has suggested that a subset of HCCs can also arise from a liver progenitor/stem cell (2). Molecular analyses of HCCs have identified various gene mutations and dysregulated signaling pathways in tumors, including alterations that up-regulate the Wnt/β-catenin, Ras/Raf/MEK/ERK, PI3K/mTOR and Sonic Hedgehog pathways (3). Gene mutations that activate the Wnt/β-catenin signaling pathway are observed in 50% of HCCs, and the most common of these is CTNNB1 mutations that result in stabilization of β-catenin (4). Thus, one approach for generating mouse models for HCC has been to activate the Wnt signaling pathway via Ctnnb1 mutation (5). Wnt pathway activation in adult murine hepatocytes fails to induce tumors (6-8). However, introduction of genetic alterations such as Ha-Ras or Akt mutation in adult hepatocytes in addition to Wnt pathway activation does result in HCC (9, 10). Published data therefore indicate that activation of the Wnt pathway alone is insufficient for HCC initiation, at least in hepatocytes.

Because the Wnt signaling pathway plays a critical role in regulating stem/progenitor cell self-renewal and because of the suggestion that a fetal progenitor is the cell of origin for some human HCCs, we hypothesized that activation of the Wnt pathway in a unique population of bipotential fetal liver cells that we have identified could give rise to HCC in vivo without the introduction of additional genetic events. As presented below, these fetal liver cells are characterized by their expression of the Cited1-CreERTM-GFP BAC transgene (11) and express CD45, in addition to markers characteristic of hepatic
stem/progenitor cells in fetal liver. They can differentiate, both in vitro and in vivo, into hepatocytes and cholangiocytes. We assessed the ability of β-catenin stabilization to transform these cells by generating mice (Tg-β-catS) that carried both the BAC transgene and a Ctnnb1 conditionally stabilized allele (Ctnnb1+ex3(fl)) (11, 12). By 26 weeks of age, 91% of Tg-β-catS mice developed hepatocellular carcinomas, demonstrating that introduction of a stabilizing Ctnnb1 mutation into a fetal liver progenitor can result in endogenous HCCs in adult mice. Hepatoblastomas and lung metastases were also observed in mutant mice.
Materials and methods

Mouse strains. Animal work was carried out in compliance with the Institutional Animal Care and Use Committee of MD Anderson Cancer Center (Houston, Texas). Cited1-CreERTM-GFP is a transgenic line carrying a BAC transgene in which expression of the Cre gene (and also a GFP reporter) is driven by a 190kb fragment 5' of the Cited1 gene, and Cre function is inducible with tamoxifen in a dose-dependent manner (11). Ctnnb1+/ex3(fl) and ROSA26R-LacZ mouse strains were also used in the study (11-13). Ctnnb1+/fl; Cited1-CreERTM-GFP embryos were generated and treated with tamoxifen (0.5mg/40g maternal body weight) at E14.5, which resulted in β-catenin stabilization in transgene-expressing cells (Tg-β-catS).

RT-PCR analysis. Conditions for RT-QPCR and primers are listed in Supplemental Methods.

Histology and immunohistochemistry (IHC). Tissues were paraformaldehyde-fixed, paraffin-embedded and assessed by H&E or IHC. For IHC, tissue sections were deparaffinized and the antigens were retrieved by boiling for 20 minutes in citrate buffer (pH 5). Antibodies used are listed in Supplemental Methods.

Western blotting. Proteins were extracted from snap-frozen tissues and western blotting was performed by standard protocols (14). Antibodies used are listed in Supplemental Methods.

Reverse phase protein array (RPPA) analysis. Protein extracts from normal livers and liver tumors were prepared and subjected to RPPA as previously described (15).
**FACS analysis.** Single cell suspensions of fetal liver (E12.5 - E17.5) from *Cited1-CreERTM-GFP* mice were prepared by homogenization of fetal livers and sorting for GFP expression using the BD FACS Aria High Speed Digital Cell Sorter. Cell suspensions from embryos with no transgene served as negative controls. The *Cited1-CreERTM-GFP* transgene is known to be expressed in fetal kidney cells (11) and kidney suspensions from *Cited1-CreERTM-GFP* mice were positive controls. Antibodies used and conditions for FACS analysis is provided in Supplemental Methods.

**Cell culture.** GFP-sorted cells from fetal liver were cultured in laminin-coated dishes for 21 days in differentiating medium (16). Cells were photomicrographed, RNA was extracted, cDNA was synthesized and semi-quantitative PCR analysis was performed as previously described (17).

**X-gal staining.** *Cited1-CreERTM-GFP; ROSA26R-LacZ* embryos were treated *in utero* at E14.5 with tamoxifen (3mg/40g maternal body weight). Livers were dissected at two months of age and stained with X-gal as described in Supplemental Methods.

**Statistical analysis.** Statistical significance of the results between groups was determined by Student’s t-test. Statistical differences were considered significant if p <0.05 (*), <0.01 (**) and <0.001 (**). All data is represented as mean ± SEM.
Results

Expression of Cited1-CreERTM-GFP transgene in fetal liver. Transgene expression was detectable by GFP fluorescence in E13.5 liver (Fig. S1, A,a). FACS analysis of liver cell suspensions (E11.5 - E17.5) showed robust GFP expression in 4% of fetal liver cells (denoted as CreERTM-GFP+) at E11.5. This declined to 0.3-0.6% by E14.5, and by E17.5 the GFP+ cells were undetectable. GFP+ cells in fetal kidney served as a control (Table 1a). In contrast to the very low frequency of GFP+ cells in livers from the BAC transgenic mice, widespread endogenous CITED1 protein expression has been observed in fetal liver from wild-type mice at E11.5 - E14.5 (18). Thus, while the transgene includes 190kb from the 5' region of the Cited1 gene, transgene expression is much more restricted than that of the endogenous locus.

CreERTM-GFP+ cells are bipotent in cell culture. Semi-quantitative PCR analysis of E12.5 and E13.5 CreERTM-GFP+ cells showed expression of albumin, α1-antitrypsin, cytokeratin 18, and vinculin, but not cytokeratin 19 (Fig. S1A, b). Because the GFP+ cells were so rare in fetal liver, we speculated that they might be progenitor cells. To test this, we isolated CreERTM-GFP+ liver cells by FACS sorting, plated them at a low density (3x10^3/cm^2), and cultured them under conditions that induce differentiation. CreERTM-GFP+ cells clonally expanded in culture and gave rise to hepatocytes and cholangiocyte-like cells (Fig. S1B, a-c). Semi-quantitative PCR analysis showed that these cultures expressed markers of hepatocyte lineage (albumin, α-fetoprotein and α1-antitrypsin), cholangiocyte markers (cytokeratin19 and vinculin) and cytokeratin 18 and 8, which were expressed by both these lineages (Fig. S1B, d), indicating that CreERTM-GFP+ cells were indeed bipotent.
Fate mapping of CreERTM-GFP+ cells in liver. We crossed Cited1-CreERTM-GFP mice with the Rosa26R-LacZ reporter mice (13). Following tamoxifen treatment (3mg/40g body weight) of embryos at E14.5, liver samples were collected at 2 months and assessed for the presence of β-gal+ cells. In the liver, hepatocytes (hexagonal large cells) and cholangiocytes of both small and large bile ducts (small cuboidal cells) were positive for β-galactosidase enzyme activity (Fig. 1A, a-c). Immunofluorescence analysis with antibody specific for β-galactosidase on liver sections showed β-gal+ cells in the liver (Fig. 1A, d). Co-staining with Sox9, a marker for adult liver progenitors (19) and β-galactosidase antibodies identified cells of bile ducts that were double positive, suggesting that the transgene-expressing cells give rise to the ductal cells (Fig. 1A, e).

Expression of cell surface markers characteristic of progenitors in the CreERTM-GFP+ cells. We characterized the fetal liver GFP+ cells at E11.5-E13.5 with markers characteristic of hepatic stem/progenitors by FACS analysis. Over 95% of the CreERTM-GFP+ cells expressed DLK1, a fetal stem/progenitor marker which is expressed between E10.5-E16.5 and is undetectable in neonatal and adult livers (20) (Fig. 1B, a). EpCAM expression was detected in 87% of GFP+ cells in E11.5 livers (Fig. 1B, a). This decreased to 49% and 35% at E12.5 and 13.5, respectively (Fig. 1B, b). Of note, all cells positive for EpCAM were also positive for DLK1 (Fig. 1B, a). The percentage of CD13+ and CD133+ cells in the CreERTM-GFP+ cells declined from 95% at E11.5, to 89% at E12.5, to 64% at E13.5 (Fig. 1B, c,d). In addition, the expression of AFP was detectable in only 3.3%, 2.5% and 13.5% of CreERTM-GFP+ cells at E11.5, E12.5 and E13.5, respectively (Fig. S2A, a,b). Table 1b summarizes these findings.
Hepatic colony forming units (h-cfus) from fetal liver are integrin α6 positive (low), integrin β1 positive, and negative for at least three hematopoietic stem cell markers (Ter119, CD45 and CD117 [c-kit]) (21). Like h-cfus, CreERTM-GFP+ cells did not express CD117 or Ter119 (hematopoietic stem cell markers), but unlike h-cfus, a significant portion (31%) did express CD45 (Fig. S2B, a,d,e). In addition, >90% of CreERTM-GFP+ cells expressed integrin β1, like h-cfus, but, unlike h-cfus, were negative for integrin α6 (Fig. S2B, a-c). About 26% of the CreERTM-GFP+ cells expressed both integrin β1 and CD45 (Fig. S2B, d,e).

Q-PCR analysis of RNA isolated from the CreERTM-GFP+ and CreERTM-GFP− cells from E12.5 fetal liver indicated that expression of Cd13, Ecadh, Lgr5 and Dlk1 was increased in CreERTM-GFP+ cells relative to CreERTM-GFP− cells (Fig. S1C).

Cited1-CreERTM-GFP; β-cat+/fl (Tg-β-catS) mice developed HCCs, HBs and lung metastases. To test the tumorigenic potential of CreERTM-GFP+ fetal liver cells, we mosaically and somatically stabilized β-catenin (β-catS) in the CreERTM-GFP+ fetal liver cells. Cited1-CreERTM-GFP and Ctnnb1+/ex3(fl) littermates served as controls. Experimental animals displayed no reduced viability, but did display hepatomegaly (Table 2). Palpatable tumors were detected as early as eight weeks and, by 26 weeks, 91% (20/22) of Cited1-CreERTM-GFP; Ctnnb1+/ex3(fl) mice (hereafter denoted Tg-β-catS) developed tumors (Fig. 2A). Mice were sacrificed when they became moribund and tumors were collected. Within an animal, multiple tumors of variable sizes were randomly distributed in all liver lobes (Fig. 2B, b). Of note, apparent lung metastases were also grossly observed in two of the 20 tumor-bearing mice that were sacrificed at 6 months of age (arrowheads, Fig. 2B, c). Genotypic analysis confirmed the presence of
the recombined Ctnnb1 allele in the tumors (Fig. 2B, d). Interestingly, loss of the wild-type allele of Ctnnb1 (marked by *) was noted in some tumors (e.g., T2).

Complete histological evaluation was carried out on liver sections from 12 tumor-bearing and control mice. Small foci of aberrant hepatocytes with increased basophilic staining was observed in most sections (Fig. 2C, b) when compared to normal liver sections (Fig. 2C, a). Nodular adenomatous lesions (Fig. 2C, c) and HCCs were most commonly present (Fig. 2C, d-f). HCCs were characterized by cytological atypia, occasional mitotic figures (Fig. 2C, g) and peliosis (arrows, Fig. 2C, d) and were very similar to human HCC.

Unexpectedly, of the 12 HCC-bearing mice, five (42%) also had histologically detectable hepatoblastomas (HBs). HBs were typically composed of undifferentiated monotypic cells, characteristic of embryonic liver cells, with scanty cytoplasm (Fig. 2C, e) and numerous mitotic figures (Fig. 2C, h). In human tumors this histology is classified as the embryonal undifferentiated subtype of HB (22). Some mice also had moderately differentiated pure fetal HB with steatosis (Table S1 & Fig. 2C, f). These observations are consistent with the fact that we targeted a progenitor population.

Histological evaluation of a lung metastasis revealed cells with increased basophilic staining, steatosis and relatively uniform nuclei (Fig. 2C, i,j). It is not clear whether the metastases arose from HCC or HB. Determining this will require further extensive evaluation.

HCCs, HBs and lung metastases showed activation of the Wnt pathway and were hyperproliferative. Western blot analysis confirmed the expression of the truncated β-
catenin (80 kDa) encoded by the exon 3-deleted Ctnnb1 gene and also glutamine synthetase (GS) in the Tg-β-cat\(^S\) HCCs (Fig. 3A). Q-RT PCR analysis also revealed a significant increase in the mRNA expression of Gs and other previously reported liver-specific Wnt target genes such as glutamate receptor 1 (Glt1), ornithine amino transferase (Oat) and Leukocyte cell-derived chemotaxin 2 (Lect2) (23, 24), in Tg-β-cat\(^S\) tumors (Fig. 3B). A statistically significant increase in expression of Axin2, c-Myc and Cyclin D1, the canonical Wnt targets, was also observed in the Tg-β-cat\(^S\) tumors (Fig. 3B).

β-catenin IHC demonstrated heterogeneous expression with increased cytoplasmic and nuclear localization in HCCs, pure fetal HBs, and lung metastasis from Tg-β-cat\(^S\) mice (Fig. 3C, a,b,d). In contrast, β-catenin staining in the embryonal undifferentiated HB was strikingly nuclear (Fig. 3C, c). In livers from littermate control mice, Wnt signaling activity, marked by the expression of GS, was restricted to a single layer of perivenular hepatocytes (Fig. S3A, b). GS immunohistochemistry revealed both an increased and an altered expression pattern in all Tg-β-cat\(^S\) HCCs in which it was uniformly expressed in all the hepatocytes of the tumor and was not restricted only to perivenular hepatocytes (Fig. 3C, e). In contrast, GS expression was heterogeneous in the pure fetal HB and lung metastasis (Fig. 3C, f,h). In the embryonal undifferentiated variant of HB, GS immunoreactivity was hardly detectable (Fig. 3C, g). In one month old Tg-β-cat\(^S\) mice that did not show any overt tumors, we detected increased cytoplasmic and nuclear localization of β-catenin, aberrant spatial expression of GS in non-perivenular areas and increased proliferation as detected by Ki67 (Fig. S3B, d-f) when compared with age-matched controls (Fig. S3B, a-c). Western blotting also confirmed
these changes (Fig. S3C). Canonical Wnt targets were also upregulated in these mice (Fig. S3D).

Tumors from Tg-β-catS mice (Fig. 3C, i-k) and the lung metastasis (Fig. 3C, l) showed increased proliferation (Ki67 staining) compared to liver sections from littermate control mice (Fig. S3B, c). The embryonal undifferentiated variant of HB displayed the highest frequency of proliferating cells (Fig. 3C, k).

**HCCs displayed activation of the MAPK and mTOR pathway.** To identify key cancer signaling pathways altered in the HCCs, we performed reverse phase protein array (RPPA) analysis and compared the protein expression profile of key proteins in Tg-β-catS HCCs and normal liver of control littermates (Fig. S4). The increased expression of mTOR and MAPKpT202 detected by RPPA suggested that two key cancer signaling pathways, Ras/Raf/MEK/MAPK and PI3K/mTOR, were significantly altered in Tg-β-catS tumors. These two pathways can be activated by FGFR1 (reviewed in (25)), and FGFR1 (pT766) was also identified by RPPA as being upregulated in the Tg-β-catS tumors (Fig. S4). Western blot analysis validated the RPPA analysis data. In the Ras/Raf/MEK/MAPK pathway, we confirmed significant upregulation of MEK1/2 (phospho), PKCα (total and pT492), ERK1/2 (total and pT202/204), c-MYC (a downstream target of the pathway), and FOX03a (pS318) (a proapoptotic gene that is inhibited by phosphorylation by ERK1/2) (Fig. 4A). In the PI3K/mTOR pathway, besides upregulation of mTOR (total and pS2448) by Western analysis, we also identified upregulation of Raptor and GβL, two TORC1 complex proteins. Interestingly, PTEN, the negative upstream regulator of the mTOR pathway whose loss results in HCC (26), was significantly downregulated in Tg-β-catS tumors (Fig. 4B). Besides these two key
signaling pathways, upregulation of stathmin, SRC, and CHK1, all of which have been associated with hepatocarcinogenesis (27-29), was detected by RPPA analysis (Fig. S4). Immunohistochemistry with PKCα (pT492) and ERK1/2 (pT202) antibodies confirmed their expression in HCC tissue (Fig. 4C, e,f). Similarly, upregulation of mTOR (pS2448) and S6 kinase (pS235/236) was detected in the HCC lesions (Fig. 4C, g,h). While the HB fetal variant showed upregulation of PKCα (pT492), ERK1/2 (pT202), mTOR (p2448) and S6 kinase (pS235/236) (Fig. 4C, m-p), the embryonal undifferentiated variant showed only upregulation of PKCα (pT492) and only a few phospho-S6 positive cells were detected (Fig. 4C, i, l), suggesting that alterations in signaling pathways were specific to the cell type comprising the tumor.

**Tumors express stem/progenitor markers.** To test if the HCCs in our model expressed stem/progenitor markers characteristic of the cells from which they arose, we performed Q-RT PCR. A statistically significant increase in the expression of Cited1, Dlk1, CD133, and Lgr5 was observed (Fig. 5A). In addition, the expression of Afp, a marker for premature hepatocytes and also a Wnt target gene in liver, and Sox9 and Sox4, markers for adult progenitors in liver, were also significantly increased in tumors (Fig. 5A). Increased Cited1, Lgr5, Sox9 and Afp was also confirmed at the protein level by Western blotting (Fig. 5B). By IHC, CITED1 expression was detected uniformly in all hepatocytes of the HCCs (Fig. 5C, a), whereas DLK1 expression was more heterogeneous (Fig. 5C, b) and SOX9 expression was nuclear and confined to small cells in tumors (arrows, Fig. 5C, c). In the embryonal undifferentiated variant of HB, CITED1 expression was weak, whereas DLK1 and SOX9 expression was robust with almost all cells expressing these markers (Fig. 5C, d,e,f). In the pure fetal HB, CITED1
expression was uniform and robust (Fig. 5C, g), high DLK1 expression was restricted to a few hepatocyte cells, (Fig. 5C, h), and SOX9 expression was nuclear and restricted to a very few cells as in the HCCs (arrows, Fig. 5C, i). CITED1 expression was seen in bile ducts (black arrows, Fig. 5D, a) and hepatocytes emerging from them (red arrows, Fig. 5D, a) and in tumors (HCCs and HBs) around bile ducts (Fig. 5D, b,c).
Discussion

We report here the identification of a novel population of fetal liver progenitor cells and the development of endogenous and metastatic liver tumors when β-catenin stabilization/Wnt pathway activation -- alone -- is targeted to these cells. These tumors also showed upregulation of the Ras/Raf/MEK/MAPK and PI3K/mTOR pathways.

Wnt pathway activation by CTNNB1 mutation is observed in 20-40% of human HCCs and 50-90% of HB (30-32). However, in mice activation of the Wnt pathway by over-expression of wild type or stable mutants of β-catenin in hepatocytes failed to result in tumor development (6, 7, 33). For tumors to develop in these mice, Wnt pathway activation had to be accompanied by additional alterations such as activation of either Ha-Ras or Akt (9, 10) or treatment with hepatotoxins (34). In this study, we hypothesized that β-catenin stabilization in an early fetal progenitor would be sufficient for tumor development. Therefore, using the Cited1-CreERTM-GFP mouse strain (11) we targeted Wnt pathway activation to CreERTM-GFP+ cells, a unique, and previously uncharacterized, early fetal liver progenitor.

Both in vitro differentiation studies and in vivo lineage tracing studies demonstrated that CreERTM-GFP+ cells are progenitors of hepatocytes and cholangiocytes in the adult liver. Thus they are functionally like fetal hepatic stem/progenitors which have been characterized, in part, by expression of cell surface markers such as DLK1, EpCAM, E-cadherin, CD13, and CD133 and which are considered to be bipotent progenitors for both hepatocytes and cholangiocytes (35). CreERTM-GFP+ cells also express DLK1, CD13, EpCAM, CD133, and E-cadherin, each of which has been reported to be a marker for fetal stem/progenitor cells. However, unlike previously characterized hepatic colony forming units (h-cfus) (16), the CreERTM-
GFP+ cells do not express integrin α6 and a fraction did express CD45 (a hematopoietic stem cell marker). Notably, the expression of AFP was detected in only a small percentage of cells. The expression of CD45 is unique to the CreER™-GFP+ cells. Recent studies in humans suggest that hepatic progenitor-like cells also express hematopoietic markers such as CD45 and CD109 (36). Additionally, co-immunofluorescence studies demonstrated that CreER™-GFP+ cells also gave rise to SOX9-expressing periductal cells in adult liver, which are considered to be adult liver progenitors. Thus the CreER™-GFP+ cells are a unique cell population which represents ~4% of the liver in E11.5 mice, a time point at which there is an increase in hepatic parenchyma and hepatoblast expansion. These cells were almost undetectable by E17.5, by which time liver development is near to completion.

In animals in which β-catenin was stabilized in the CreER™-GFP+ cells (Tg-β-catS mice), over 90% developed HCC by 26 weeks. This is in striking contrast to the absence of tumors when β-catenin is stabilized in adult hepatocytes using a Cre-expressing adenovirus or using a adolaseB promoter-driven transgene expressing a mutant stable β-catenin protein (7). Our conditional genetic model using the Tg-β-catS mice demonstrates that the CreER™-GFP+ liver stem/progenitor cells can be transformed by just β-catenin activation. Only a small fraction of CreER™-GFP+ cells express AFP. Future studies will be required to determine whether the tumors in the Tg-β-catS mice arise from the AFP+ and/or the AFP− fraction of the CreER™-GFP+ cells. Of note, activation of Wnt signaling pathway in fetal hepatoblasts using an Afp-driven Cre transgene is embryonic lethal, making it impossible to evaluate potential tumorigenesis of the large population of Afp+ fetal liver progenitors (37).
Like human HCCs, the $Tg$-$\beta$-cat$^S$ tumors histologically showed nuclear atypia, mitotic figures, and peliosis, and robustly and heterogeneously expressed GS and nuclear $\beta$-catenin. Approximately ~40% of mice that developed HCCs also developed HBs. In the $Tg$-$\beta$-cat$^S$ mice, $\beta$-catenin was stabilized at ~E14.5, at which time hepatoblast lineage bifurcation begins. Because of this, it is possible that $\beta$-catenin stabilization occurred in a heterogeneous population of fetal progenitors, some of which were more differentiated than others. This is fully consistent with the occurrence of both HCC and HB in these mice. Like human HBs that are classified into distinct classes based on their histology (22), we observed two distinct histologies: a pure fetal moderately differentiated HB and an embryonal undifferentiated HB. Some mutant mice showed both histologies of HB. Like human HBs, both types of murine HBs displayed increased nuclear $\beta$-catenin expression, homogeneously in the embryonal HBs and heterogeneously in the fetal histology HBs. GS expression in the fetal moderately differentiated HBs was similar to that in the HCCs, although more heterogeneous. GS expression was lower in the embryonal undifferentiated HBs, consistent with the expression of GS in more differentiated cells of the liver.

Lung metastases were grossly observable in some $Tg$-$\beta$-cat$^S$ mice, an unusual occurrence in mouse models. 46% and 44% of human HCC and HB patients, respectively, have metastatic disease, primarily in the lung (38, 39). As was the case for the primary $Tg$-$\beta$-cat$^S$ tumors, we observed robust $\beta$-catenin expression in a lung metastasis.

The observation of HCCs and sometimes, additionally, HBs, raises a question about the cell type(s) from which these tumors arose. HBs are thought to arise from
maturation arrest of infant liver stem cells and HCCs to develop from more differentiated cells that are nevertheless from the same lineage (40, 41). For human HCC, a fetal cell origin has been suggested for a subset of tumors based on their fetal liver gene expression profile, and the development of HCCs following \textit{ex vivo} genetic manipulation of murine hepatoblasts is consistent with this (42, 43). Alternatively, HCCs may arise following dedifferentiation of adult hepatocytes and re-expression of fetal markers during the course of malignant transformation (2). The recent observation of CITED1 expression in regenerating hepatocytes following partial hepatectomy or DDC treatment (18) suggests that fetal markers can be re-expressed in adult hepatocytes during regeneration.

Another possibility is that the HCCs arose from the fetal CreER\textsuperscript{TM}-GFP\textsuperscript{+} cell-derived SOX9\textsuperscript{+} adult progenitors that we identified in our mice. Examination of the livers of \textit{Tg-\beta-cat}\textsuperscript{S} mice revealed that small HCCs were almost invariably located around bile ducts, similar to the localization of the SOX9\textsuperscript{+} stem/progenitors, consistent with the notion that tumors may have arisen from adult progenitors.

Activating mutations of the Wnt signaling pathway are very common in both mouse and human HCC (30), and in human tumors Wnt target genes, both canonical and liver-specific targets, are upregulated (23, 24, 44, 45). We observed increased cytoplasmic and nuclear localization of \(\beta\)-catenin in the liver of young (one month old) \textit{Tg-\beta-cat}\textsuperscript{S} mice and also in hepatocytes in the \textit{Tg-\beta-cat}\textsuperscript{S} HCCs. Notably, both liver and HCCs also showed an increase in both canonical (\textit{c-Myc, Cyclin D1} and \textit{Axin2}) and non-canonical Wnt targets (\textit{Gs, Glt1, Oat} and \textit{Lect2}). Such induction of canonical Wnt targets was not observed in a model in which stabilized \(\beta\)-catenin was expressed in
hepatocytes, nor did these mice develop tumors (7). These differences between the two models suggest that the ability of Wnt pathway activation to result in HCCs critically depends on the activation of canonical Wnt targets.

_Tg-β-cat^S_ tumors also expressed FGFR1, commonly observed in human HCCs, and displayed activation of the Ras/Raf/MAPK and the PI3K/AKT/mTOR pathways, both of which can be activated by FGFR1 and are present in human HCCs characterized by aggressive behavior (46). Specifically, PKCα, which has been reported to contribute to HCC proliferation, migration, and invasion via activation of MAPK (47, 48), was upregulated in _Tg-β-cat^S_ tumors as was pMEK1/2 and pERK1/2. Phospho-FOXO3A, a proapoptotic protein which is negatively regulated via phosphorylation by pERK1/2, was increased. PTEN was downregulated in tumors, whereas components of the TORC1 complex, p-mTOR, RAPTOR, and GβL, were upregulated, consistent with the known role of PTEN in negatively regulating the mTOR pathway. As noted previously, tumors displayed an increase in both liver-specific and canonical β-catenin targets. One, c-MYC, is also an effector of the MEK/ERK pathway, and another, GS, has been reported to enhance tumor growth by activation of the mTOR pathway (49, 50). Thus the activation of the Wnt pathway engineered into the CreERTM-GFP^+_ fetal liver progenitors may have not only initiated tumorigenesis, but may have also enhanced the effect of mTOR and MEK/ERK pathway activation. We also observed up-regulation of Stathmin, SRC, and CHK1, which have also been implicated in hepatocarcinogenesis (27-29).

By RPPA analysis, _Tg-β-cat^S_ HCCs uniformly displayed activation of the mTOR and MEK/ERK pathways and this was subsequently verified by IHC. In contrast to HCCs, we observed distinct patterns of protein/phospho-protein expression in the two
histologic types of HBs. The fetal HB corresponded more closely to HCC with upregulation of PKCα, ERK1/2, mTOR and S6 proteins, whereas the embryonal undifferentiated HB showed significant upregulation of only PKCα. The expression of β-catenin was also distinct in these two histologies of HB, with the embryonal undifferentiated HB displaying strikingly nuclear localization and the pure fetal HB resembling the HCCs. These results suggest that the specific signaling pathways altered in the tumors are dependent upon the cell type within the tumor.

In summary, we have identified a unique population of progenitor cells in fetal liver which are marked by the expression of the Cited1-CreER\textsuperscript{TM}-GFP transgene. Somatic β-catenin stabilization in these cells results in the frequent development of both HCCs and HBs with spontaneous lung metastases. Our model provides valuable evidence for a common origin of HCC and HB from the Tg-β-cat\textsuperscript{S} stem/progenitor cell. This model will be extremely valuable for understanding the pathobiology of human HCCs, in particular those that display an especially poor prognosis and are thought to be of stem/progenitor origin.
References


TABLES

Table 1a. FACS sorting of embryonic liver and kidney samples for GFP expression.

<table>
<thead>
<tr>
<th></th>
<th>Liver (% GFP positive)</th>
<th>Kidney (% GFP positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E 11.5</td>
<td>2.5 – 4.0%</td>
<td>not determined</td>
</tr>
<tr>
<td>E 12.5</td>
<td>0.8 – 1.0%</td>
<td>not determined</td>
</tr>
<tr>
<td>E 13.5</td>
<td>0.3 – 0.6%</td>
<td>not determined</td>
</tr>
<tr>
<td>E 14.5</td>
<td>0.3 -0.1%</td>
<td>0.5%</td>
</tr>
<tr>
<td>E 17.5</td>
<td>undetectable</td>
<td>0.9%</td>
</tr>
</tbody>
</table>

*Expressed as percentage of total liver cells

Table 1b. Expression of hepatoblast markers in CreER<sup>TM</sup>-GFP<sup>+</sup>cells.

<table>
<thead>
<tr>
<th></th>
<th>E11.5</th>
<th>E12.5</th>
<th>E13.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD13</td>
<td>95</td>
<td>91</td>
<td>85</td>
</tr>
<tr>
<td>CD133</td>
<td>90</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>CD13/CD133</td>
<td>90</td>
<td>89</td>
<td>83</td>
</tr>
<tr>
<td>DLK1</td>
<td>98</td>
<td>95</td>
<td>97</td>
</tr>
<tr>
<td>EpCAM</td>
<td>78</td>
<td>49</td>
<td>21</td>
</tr>
<tr>
<td>DLK1/EpCAM</td>
<td>77</td>
<td>49</td>
<td>21</td>
</tr>
<tr>
<td>AFP</td>
<td>4</td>
<td>2</td>
<td>14</td>
</tr>
</tbody>
</table>

*Expressed as percentage of CreER<sup>TM</sup>-GFP<sup>+</sup> cells
Table 2. Liver weight/body weight ratio in $Tg$-$\beta$-$cat^{a}$ mice at one month of age

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Liver weight/body weight ratio</th>
<th>No. of mice analyzed</th>
<th>Fold change</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Tg$-$\beta$-$cat^{a}$</td>
<td>0.1550± 0.025</td>
<td>8</td>
<td>3.03</td>
</tr>
<tr>
<td>Controls</td>
<td>0.0511± 0.002</td>
<td>9</td>
<td>1</td>
</tr>
</tbody>
</table>

Controls vs $Tg$-$\beta$-$cat^{a}$ p<0.0005
Figure legends

**Figure 1. Characterization of CreER<sup>TM</sup>-GFP<sup>+</sup> cells in fetal liver.** A. P60 liver sections showing X-gal<sup>+</sup> liver cells, hepatocytes and bile ducts (arrows, a), higher magnification showing the small (arrows, b) and large bile ducts (c). Antibody staining of cryosections (P60) for β-galactosidase in hepatocytes (arrow, d) and in SOX9 positive (red nuclei) bile duct cells (e). 1’ and 1” show higher magnification. Scale bar, 160 µm (a, d) and 32 µm (e). B. FACS analysis for DLK1 and EPCAM (a,b), CD13 and CD133 (c,d) in the CreER<sup>TM</sup>-GFP<sup>+</sup> cells at E11.5 - E13.5.

**Figure 2. Development of liver tumors in Tg-β-cat<sup>+</sup> mice.** A. Kaplan-Meier analysis of tumor incidence in mutant animals (dotted line) and littermate controls (solid line). B. Gross appearance of normal liver (a), liver with multifocal tumors in Tg-β-cat<sup>+</sup> mice (b), and lung metastases (arrowheads, c). Representative PCR from Tg-β-cat<sup>+</sup> mice showing wildtype/floxed bands in tail and liver DNA of control mice and mutant tumors (d). Asterisk (*) indicates loss of the wildtype allele in T2. C. Histological analysis of liver tumors (b-h) and associated lung metastasis (i,j). H&E staining of normal liver (a), altered hepatocytes (denoted by arrow, b) adenoma (demarcated by dotted line, c) HCC (d-f), HB embryonal (e) and HB pure fetal (f). Insets in (a) and (b) provide higher magnification (3X). Mitotic figures of HCC (g) and embryonal undifferentiated HB (h). Lung metastasis (dotted lines, i), and higher magnification (j). Scale bar, 80 µm (a,b,d,j), 500 µm (c,e,f), and 1280 µm (i).

**Figure 3. Activation of Wnt pathway in Tg-β-cat<sup>+</sup> tumors.** A. Western blot analysis of Tg-β-cat<sup>+</sup> tumors and normal control livers for β-catenin and glutamine synthetase (GS). β-Actin and Ponceau stained membrane (P) were used as loading controls. B. Q-PCR
analysis of liver-specific β-catenin target genes (Gs), glutamate transporter 1 (Glt1), ornithine aminotransferase (Oat) and Lect2 in Tg-β-cat\textsuperscript{S} tumors (n=3) and canonical target genes Axin2, c-Myc, and CyclinD, compared to littermate normal livers (n=3). C. β-catenin IHC in HCC (a), HB pure fetal (b), embryonal undifferentiated HB (c), and lung metastasis (d). GS staining in HCC, fetal HB, embryonal HB, and lung metastasis (e-h). Ki67 staining in HCC, HB, and metastasis (i-l). Scale bar, 80 µm.

**Figure 4. Activation of the MAPK and mTOR pathways in Tg-β-cat\textsuperscript{S} tumors.** A, B. Western blot analysis of MAPK and mTOR pathway components in HCC from Tg-β-cat\textsuperscript{S} HCCs compared to control livers. GAPDH and Ponceau stained membranes were used as loading controls. C. IHC analysis of phospho-PKCα (a,e,l,m), phospho-ERK1/2 (b,f,j,n), phospho-mTOR (c,g,k,o), and pS6 (d,h,l,p) in the normal liver (a-d), HCCs (e-h), embryonal HB (i-l) and fetal HB (m-p) of Tg-β-cat\textsuperscript{S} mice. Insets show higher magnification (e-p). Necrotic areas denoted by *. Scale bar, 80µm.

**Figure 5. Expression of hepatic stem/progenitor markers in Tg-β-cat\textsuperscript{S} tumors.** A. RT-PCR analysis of HCCs (n=3) and control livers (n=3) for expression of stem/progenitor markers. B. Western blot analysis of stem/progenitor markers in Tg-β-cat\textsuperscript{S} HCCs. C. IHC showing expression of CITED1 (a,d,g), DLK1 (b,e,h), and SOX9 (arrowheads, c, i and f) in HCC (a-c) embryonal HB (d-f) and fetal HB (g-i). Scale bar, 70 µm. D. IHC showing CITED1 expression in bile duct epithelium (black arrows, a) and in hepatocytes emerging around the bile ducts (red arrows, a). Tumors (HCC, b and HB, c) originating around the bile ducts. Insets in (a) and (b) show CITED1 expression in bile duct epithelium. Scale bar; a,b, 70 µm; c, 160 µm.
Figure 1

A

B

E11.5  E13.5

DLK1

EpCAM

% of GFP positive cells

E11.5  E12.5  E13.5

DLK1+  EpCAM+  DLK1+EpCAM+

C

CD13

CD133

% of GFP positive cells

E11.5  E12.5  E13.5

CD13+  CD133+  CD13+CD133+
Figure 2
Figure 3
Author Manuscript Published OnlineFirst on May 21, 2014; DOI: 10.1158/0008-5472.CAN-13-3275
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

CITED1

DLK1

SOX9

HCC

Embryonal HB

Fetal HB

Tg-β-cat

CITED1
β-catenin activation in a novel liver progenitor cell type is sufficient to cause hepatocellular carcinoma and hepatoblastoma

Sharada Mokkapati, Katharina Niopek, Le Huang, et al.

Cancer Res  Published OnlineFirst May 21, 2014.

Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-13-3275

Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/06/03/0008-5472.CAN-13-3275.DC1

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Sign up to receive free email-alerts related to this article or journal.

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/early/2014/05/21/0008-5472.CAN-13-3275. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.