Forced activation of β-catenin signaling supports the transformation of hTERT-immortalized human fetal hepatocytes

Running Title: Transformation of immortalized human hepatocytes

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Keywords: Hepatocellular carcinoma; hepatocarcinogenesis; telomerase; β-catenin; thrombospondin; translocations.
List of Abbreviations: HCC; hepatocellular carcinoma; hTERC, human telomerase RNA component; hTERT, human telomerase reverse transcriptase; APC, adenomatous polyposis coli; GSK-3β, glycogen synthase kinase-3β; CRT, β-catenin-related transcription; PAC, puromycin-N-acetyltransferase; PD, population doubling; qPCR, real-time quantitative PCR; RQ-TRAP, real-time quantitative telomeric repeat amplification protocol; RTA, relative telomerase activity; TCF/LEF, T-cell factor/lymphoid enhancing factor; DAPI, 4′,6-diamidino-2-phenylindol; S100A4, S100 calcium binding protein A4; SD, standard deviation; SH ble, Zeocin resistance gene; shRNA, small-hairpin RNA; siRNA, small-interfering RNA; mFISH, multicolor karyotyping-fluorescence in situ hybridization; MMP, matrix metalloproteinases; PLAU, urokinase-type plasminogen activator; VEGFA, vascular endothelial growth factor A; IGF, insulin-like growth factor; THBS1, thrombospondin 1.
Abstract

Hepatocarcinogenesis is a multistep process driving the progressive transformation of normal liver cells into highly malignant derivatives. Unlimited proliferation and telomere maintenance have been recognized as prerequisites for the development of liver cancer. Moreover, recent studies identified illegitimate β-catenin signaling as relevant hit in a considerable subset of patients. To further investigate the currently not well-understood malignant evolution driven by telomerase and β-catenin, we monitored cytogenetic and phenotypic alterations in untransformed telomerase-immortalized human fetal hepatocytes following forced activation of β-catenin signaling. As expected, constitutive activation of β-catenin signaling significantly enhanced proliferation with decreasing serum dependence. Previously intact contact inhibition was almost completely eliminated. Interestingly, after several passages in cell culture, immortalized clones with dominant positive β-catenin signaling acquired additional chromosomal aberrations, in particular translocations, anchorage-independent growth capabilities, and formed tumors in athymic nude mice. In further support for the driving role of β-catenin during hepatocarcinogenesis, improved colony growth in soft agar and accelerated tumor formation was also confirmed in Huh7 cells following stable expression of the constitutively active S33Y β-catenin mutant. Telomerase inhibition demonstrated that short-term expansion of transformed clones was not telomerase-dependent. Finally, cancer pathway profiling in derived tumors revealed upregulation of characteristic genes associated with invasion and angiogenesis. In conclusion, illegitimate activation of β-catenin signaling enhances the transformation from immortalization to malignant growth in human fetal
hepatocytes. Our data functionally confirm a permissive role for β-catenin signaling in the initial phase of hepatocarcinogenesis.
Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer mortality worldwide with continuously rising incidence rates in Western countries (1, 2). Although the principal clinical risk factors for HCC are well defined, molecular mechanisms contributing to tumor initiation and early progression are still not completely understood. In almost 90% of human malignancies, telomerase activation, characteristically mediated by re-expression of the rate-limiting catalytic subunit human telomerase reverse transcriptase (hTERT), has been observed as early event (3). Telomerase is a cellular RNA-dependent DNA polymerase responsible for telomere maintenance and stabilization (4). Telomeres are protective nucleoprotein structures at the end of linear chromosomes (5). In normal somatic cells, hTERT is suppressed and consequently telomerase not active. Due to the end-replication problem, telomeres progressively shorten with every cell division until a critical length is reached and the cells enter senescence, a post-mitotic quiescent state (6). Interestingly, several studies have recently demonstrated telomerase activation already in precancerous hepatic lesions (7-10). Based on these findings, telomerase activation has been proposed as an early prerequisite in hepatocarcinogenesis (11, 12). In addition to a limitless replicative potential (immortalization), further genetic alterations are required for self-sufficiency in growth signals, insensitivity to anti-growth signals, effective evasion of apoptosis, and altered differentiation (13).

In a considerable subset of patients with HCC, Wnt/β-catenin signaling is activated (14). This pathway is, for example, crucial for the expansion and survival of
embryonic hepatoblasts (15, 16). Recently, animal models also revealed that mobilization of hepatic stem cells during liver regeneration is associated with activation of Wnt/β-catenin signaling (17, 18). β-Catenin is the key signaling molecule of the pathway. In the absence of activating ligands, a destruction complex consisting of axin, adenomatous polyposis coli protein (APC), and glycogen synthase kinase-3β (GSK-3β), phosphorylates β-catenin in the cytoplasm. Phosphorylated β-catenin undergoes degradation. In the activated state, Wnt binds to the membrane receptor frizzled and induces the dissociation of the destruction complex. β-Catenin escapes degradation and accumulates in the cytoplasm leading to increased translocation into the nucleus, where β-catenin initiates the expression of various target genes (17, 19, 20). Activation of Wnt/β-catenin signaling, primarily caused by inactivating mutations in APC, AXIN1, or CTNNB1 (21, 22), has recently been identified as a permissive pathway driving downstream transformation events in colorectal cancer (23).

Interestingly, compared to the subgroup with TP53 mutations, liver cancers with activating mutations in CTNNB1 have relatively little chromosomal instability (24), suggesting a driving role for this pathway also in hepatocarcinogenesis.

In this study, we employed previously characterized telomerase-immortalized human fetal hepatocytes (FH-hTERT) as cell culture model for untransformed proliferating human liver cells (25, 26). To analyze the functional consequences of an aberrantly activated β-catenin-related transcription (CRT), we monitored cancer cell characteristics and transformation events in FH-hTERT following forced activation of β-catenin signaling.
Material and Methods

Cell lines and plasmids

The local ethics committee approved utilization of FH-hTERT cells (approval number OB-034/06). FH-hTERT clones were used at population doubling (PD) 35 to 40 (just bypassing the senescence checkpoint) and 80 to 100. As described before, FH-hTERT did not display a malignant phenotype (25). As control human liver cancer cell lines, we cultured β-catenin-positive HepG2 and a Huh7 clone with a low baseline β-catenin expression (Supplementary Fig. S1). Expression vectors for the wild-type and the constitutively active S33Y β-catenin mutant (pbcatwt and pbcatS33Y, respectively) were kindly provided by H. Clevers (Hubrecht Laboratory, Utrecht, Netherlands). The backbone vector pcDNA (Invitrogen, Karlsruhe, Germany) was transfected as vector control.

Cell culture and transfection

All cells were cultured in Dulbecco’s Modified Eagle Medium with high glucose (4.5 g/L), 10% inactivated fetal bovine serum, and antibiotics (Invitrogen). For FH-hTERT, culture medium was additionally supplemented with 5 μg/mL insulin and 2.4 μg/mL hydrocortisone (Sigma-Aldrich, Seelze, Germany). Cells were nucleofected using Nucleofector Solution V (Amaxa, Köln, Germany) and program T30, and subsequently selected with Zeocin (Invitrogen) at 200 μg/mL for FH-hTERT and 50 μg/mL for Huh7 cells.
Plasmid expression analysis and immunoblot

To quantitate β-catenin expression, RNA was extracted with RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse transcribed with ThermoScript RT-PCR Systems (Invitrogen). For real-time quantitative PCR (qPCR), we employed a validated primer set for CTNNB1 together with various internal reference genes (GAPDH, RPL13A, B2M, TBP) as basket housekeeper (QuantiTect Primer Assay; Qiagen). Amplification was carried out with QuantiTect SYBR Green PCR Master Mix (Qiagen) on an ABI Prism 7900H thermal cycler (Applied Biosystems, Foster City, CA). PCR efficiency ($E$) was determined for each amplicon. Finally, expression levels were derived by an efficiency-corrected model and comparative quantification ($E^{\Delta\text{Ct}[\text{CTNNB1}]} / E^{\Delta\text{Ct}[\text{reference genes}]}$) (27). In addition, cell lysates were extracted and quantified with the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). SDS-PAGE was conducted with 20 µg protein per lane. Following blotting, nitrocellulose membranes were blocked with 5% non-fat dry milk. Primary antibody solution detecting β-catenin (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) was incubated at 4°C overnight. Incubation with species-specific secondary antibody solution (1:1000; Santa Cruz Biotechnology) conjugated with horseradish peroxidase was performed for 45 minutes at room temperature. Detection was achieved with the ECL Western Blotting Analysis System (Amersham Biosciences, Freiburg, Germany).

Dual-luciferase reporter assay

CRT reporter plasmids (Super8XTOPFlash and Super8XFOPFlash) were kindly provided by R.T. Moon (Howard Hughes Medical Institute, Seattle, WA). The TOPFlash plasmid (M50) contains T-cell factor/lymphoid enhancing factor (TCF/LEF) binding sites driving the expression of Firefly luciferase. The control plasmid
FOPFlash (M51) carries mutant TCF/LEF binding sites. As co-reporter vector, pRL-CMV with CMV-driven expression of *Renilla luciferase* (Promega, Madison, WI) was used to monitor transfection efficiency. Cells were transfected by lipofection with JetPEI (Polyplus, Illkirch Cedex, France) according to instructions from the manufacturer. Two days after transfection, cells were harvested and assayed using the Dual-Luciferase Reporter Assay System (Promega). CRT was expressed as luciferase activity ratio M50/M51, following correction for transfection efficiency (M50/pRL-CMV and M51/pRL-CMV).

**Cell proliferation tests**

To monitor cell proliferation, we performed serial cell counts in a standard hemocytometer. Additionally, we employed the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) seeding 5 x 10³ cells per well in 96-well plates. At each time-point, an entire column (n = 8) was assayed and the mean OD value was calculated. ΔOD was obtained by comparing the reading to the mean OD obtained 12 hours after seeding.

**Serum dependence and contact inhibition**

To investigate serum dependence, cells were seeded in 96-well plates and serum was omitted in the following medium changes. Proliferation was monitored as described above. Cell cycle profiles of near-confluent (40-60%) and super-confluent cultures (culture for 3 days after reaching confluency as determined by phase-contrast microscopy) were obtained by incubating fixed cells in propidium iodide solution (working concentration 25 μg/ml) containing RNase A (500 U/mL) at 37°C for 30 minutes before evaluation on a FACSCanto flow cytometer (Becton Dickinson,
Heidelberg, Germany). Cell cycle distribution of cells was determined using ModFit (Verity Software House, Topsham, ME).

**Cytogenetic investigations**

Multicolor karyotyping-fluorescence in situ hybridization (mFISH) was performed using the 24XCyte color kit for human chromosomes (MetaSystems, Altlussheim, Germany) according to the supplier’s recommendations and as previously described (25). We employed the Genome-Wide Human SNP Array 6.0 and the Genotyping Console Software 4.0 (Affymetrix, High Wycombe, United Kingdom) to assess micro-amplifications and deletions in DNA samples obtained from transformed cells in comparison to the HapMap reference (Affymetrix) and untransformed counterparts. Array analyses were performed in the Array Service Center of the Department of Clinical Chemistry, University Medical Center Hamburg-Eppendorf.

**Anchorage-independent growth**

Soft agar colony-assays were performed as previously summarized (26).

**Tumor formation in nude mice**

Animal experimentation was approved by the local review board (protocol number 25/06). All animals received humane care. Tumorigenicity was assessed by inoculating \(2 \times 10^6\) cells in 100 µL culture medium with 2% serum and mixed with 100 µL Matrigel (Sigma-Aldrich) subcutaneously into the dorsal flanks of 8- to 12-week-old NMRI athymic nude mice (Animal Facility, University Medical Center Hamburg-Eppendorf). Tumor formation was observed for a minimum of 12 months and
measured three times per week using a caliper. Tumor volume was estimated as \( (D^2 \times d)/2 \), where \( D \) is the large diameter, and \( d \) the smaller diameter of the tumor.

**Tumor genotyping**

For genotyping, tumor DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen). Amplification of specific sequences of the selection markers enclosed in the two expression plasmids introduced in our cell clones (puromycin N-acetyltransferase [PAC] and Zeocin resistance gene [SH ble]) was performed using Platinum PCR SuperMix (Invitrogen). Primer sequences are as follows: β-actin \((ACTB) \) GCC ATC CTC ACC CTG AAG TA and GTC AGG CAG CTC GTA GCT CT, PAC ACC GAG CTG CAA GAC CTC TTC CTC and CCA GGA GGC CTT CCA TCT GTT G, and \( SH \) ble GGA CTT CGT GGA CGA C and CAC GAC CTC CGA CCA CTC. In addition to tumor samples, DNA from Huh7 (negative control), FH-hTERT (PAC positive control), FH-hTERT pbcatS33Y (SH ble positive control), and mouse liver cells (background control) were analyzed.

**Immunofluorescence of tumor samples**

To visualize human cells within the observed tumors, cryostat sections were immunostained with a human-specific antibody recognizing the nuclear antigen SP-100 (kindly provided by H. Will, Heinrich-Pette-Institute, Hamburg, Germany). Sections were fixed in acetone for 10 minutes. After blocking sections in 1% bovine serum albumin solved in TRIS-buffered saline (pH 7.5) with 0.05% Tween-20 for 1 hour, the primary antibody was applied with a dilution of 1:400 for 1 hour. A species-specific fluorescence-labeled secondary antibody (Alexa Fluor 488; Invitrogen) was
employed for visualization. Finally, sections were counterstained with 4′,6-diamidino-2-phenylindol (DAPI) and examined under a fluorescence microscope.

**Telomerase inhibition**

RNA interference was utilized to suppress telomerase function. Via nucleofection (Nucleofector Solution V, program T30) a small interfering RNA (siRNA) corresponding to the essential RNA component of the human telomerase holoenzyme (hTERC; sc-106994; Santa Cruz Biotechnology) was introduced to knock-down hTERC expression and telomerase activity. To inhibit potential non-canonical hTERT functions, hTERT expression was targeted employing a small hairpin RNA (shRNA; sc-156050-SH; Santa Cruz Biotechnology) and lipofection. A lipofection protocol was required to knock-down hTERT, since nucleofection induced hTERT transcription. Expression of hTERC and hTERT were determined 48 hours following transfection. For the qPCR protocol described above, a validated primer set for hTERT (sc-156050-PR; Santa Cruz Biotechnology), a self-designed and optimized primer set for hTERC (GCCTTCCACCGTTCATTCTA and GGCZGACAGAGCCCAACTC), and the basket housekeeper were used. Telomerase activity in extracts from 1,000 cells was measured and expressed as relative telomerase activity (RTA) in comparison to telomerase-positive 293T cells with the real-time quantitative telomeric repeat amplification protocol (RQ-TRAP) as outlined before (28).

**Cancer pathway profiling**

DNase-treated RNA from FH-hTERT pcDNA, FH-hTERT pbcatS33Y, and 4 independent tumor samples, extracted with RNeasy Mini Kit (Qiagen), was reverse
transcribed with ThermoScript RT-PCR Systems (Invitrogen). After diluting the cDNA synthesis reaction with ddH$_2$O (1:5.55), expression profiling was performed with the RT$^2$ Profiler PCR Array system (SABiosciences, Frederick, MD) on an ABI Prism 7900H thermal cycler (Applied Biosystems). Primer sets on the array represent biological pathways involved in transformation and tumorigenesis. Additionally, the panel includes a set of reference genes as well as RNA and qPCR quality controls. Expression levels were determined by comparative quantification ($2^{-\Delta\Delta Ct}$) employing FH-hTERT pcDNA as calibrator (expression level = 1).

**Statistical analysis**

All experiments were performed in triplicates and with two to three repetitions. Data are presented as mean ± standard deviation (SD). The unpaired Student’s $t$-test was used for statistical analysis and $P$ values less than 0.05 were considered statistically significant.
Results

Expression of dominant positive β-catenin

FH-hTERT display low Wnt/β-catenin pathway activity as determined with a dual-luciferase reporter assay measuring β-catenin regulated gene expression (CRT). Incubation with 40 mM LiCl, a GSK-3β inhibitor and chemical pathway activator, transiently enhanced CRT and demonstrated the possibility to activate β-catenin signaling in our cell clones (data not shown). Transfection of FH-hTERT with the constitutively active S33Y β-catenin mutant (pbcatS33Y) as well as with the wild-type control (pbcatwt), resulted in a significant increase in β-catenin transcripts determined by qPCR (Fig. 1A). Following selection and expansion of Zeocin resistant cells, increased protein levels of β-catenin were observed in cells transfected with either of the two β-catenin expression plasmids. In comparison, only a faint β-catenin band was observed in FH-hTERT transfected with pcDNA (Fig. 1B). In FH-hTERT stably transfected with pbcatS33Y (FH-hTERT pbcatS33Y), increased β-catenin levels resulted in a robust upregulation in downstream pathway activity (Fig. 1C). CRT levels were not substantially increased in FH-hTERT pbcatwt, because the wild-type β-catenin is not translocated into the nucleus and does therefore not lead to target gene activation.

Forced β-catenin activation induces cancer cell stigmata

To functionally characterize phenotype alterations induced by forced β-catenin activation, we monitored proliferation, serum dependence, and contact inhibition. In comparison to FH-hTERT pbcatwt, forced β-catenin activation (FH-hTERT
pbcatS33Y) resulted in an accelerated cellular growth. This was confirmed by serial cell counts (Fig. 2A) and a photometric assay monitoring proliferation more closely (Fig. 2B). We also cultured the clones with 2% serum. Three days after seeding, we detected a significant difference in cell density between FH-hTERT pbcatS33Y and FH-hTERT pbcatwt. This difference reflects the accelerated growth in FH-hTERT pbcatS33Y (see above). However, serum-free culture significantly reduced cellular growth in FH-hTERT pbcatwt (Fig. 2C), with a 20% reduction in cell density compared to cells cultured with 2% serum for three days. In contrast, serum depletion did not significantly affect cellular growth in FH-hTERT pbcatS33Y (Fig. 2C). To determine changes in contact inhibition, we compared S-phase fractions in confluent cultures (3 days after reaching 100% confluency) with log-proliferating cultures (40-60% confluency). In FH-hTERT pcDNA, the S-phase fraction was reduced by more than 60% in confluent cultures compared to near-confluent cultures, which demonstrates effective growth inhibition by cell-to-cell contacts. In FH-hTERT pbcatS33Y, contact inhibition was almost completely eliminated, i.e., the S-phase fraction was reduced by only 10% (Fig. 2D).

**Clones with enhanced β-catenin signalling acquired translocations**

Previous cytogenetic investigations demonstrated that FH-hTERT maintained a rather intact karyotype and did not acquire clonal structural chromosomal aberrations in long-term expansion culture (25). In this study, cytogenetic monitoring of FH-hTERT pbcatS33Y revealed acquisition of translocations, which were not observed in FH-hTERT control cells. Employing mFISH, 37 metaphase spreads were analyzed. 19 metaphases showed the translocations t(5;19)(q31;p11orq11) and t(10;11)(p13;q21) (Fig. 3A). In addition, non clonal structural and numerical
abnormalities were detected in 25 metaphases. Further analyses based on SNP-arrays exposed additional micro-amplifications and deletions on almost all chromosomes (Fig. 3B). Taken together, these data suggest that FH-hTERT pbcatS33Y at least maintained the ability to proliferate despite chromosomal instability and potentially promoted the acquisition of structural chromosomal abnormalities.

**Telomerase and β-catenin support a transformed phenotype**

Anchorage-independent growth is an established *in vitro* marker for a malignant phenotype. Interestingly, after a few additional passages in cell culture, FH-hTERT pbcatS33Y developed the ability to generate colonies in soft agar (Table 1). Anchorage-independent growth was not observed in FH-hTERT pcDNA and FH-hTERT pbcatwt. In addition, early passage FH-hTERT (PD 35 to 40, immediately after bypassing the senescence checkpoint) treated with pbcatS33Y also developed anchorage-independent growth capabilities; however, with a significantly lower frequency (Table 1). To confirm the association between β-catenin activation and anchorage-independent growth, we also scored colony formation in Huh7 cells with and without illegitimate β-catenin activation. In this HCC cell line, expression of pbcatS33Y significantly induced colony growth in comparison to control cells. It should be noted, that colony formation was observed in malignant Huh7 cells after approximately 2 weeks, whereas colonies were seen in FH-hTERT pbcatS33Y only after a significantly longer latency period of seven weeks (Table 1).

As gold-standard proof for a transformed phenotype, FH-hTERT pbcatS33Y were transplanted subcutaneously into athymic nude mice. We transplanted Huh7 cells as
a positive control. Tumor formation data is listed in Table 2. As expected and as previously shown (26), no tumor formation was detected in mice transplanted with control cells and FH-hTERT pbcatwt during a one-year observation period. However, after a mean latency period of 19.0 ± 3.0 weeks, tumors formed in all FH-hTERT pbcatS33Y transplantation sites (Fig. 4A). Notably again, tumors formed with a significantly longer latency period compared to mice transplanted with Huh7 cells (Table 2). As further support for the oncogenic potential of forced β-catenin activation, tumor formation was also significantly accelerated in Huh7 cells following expression of pbcatS33Y (3.4 ± 0.9 weeks vs 7.7 ± 2.6 weeks, \( P < 0.001 \)). Huh7 pbcatS33Y cells reached a tumor size of approximately 0.8 cm\(^3\) in less than three weeks. In contrast, FH-hTERT pbcatS33Y tumors grew less aggressively (Fig. 4B). PCR genotyping and immunofluorescence employing a human-specific antibody for the nuclear antigen SP-100 clearly established that the observed tumors in mice transplanted with FH-hTERT pbcatS33Y and secondary cell lines derived from these tumors originate from transplanted human cells (Supplementary Fig. S2 and S3).

Short-term expansion of FH-hTERT pbcatS33Y was not influenced by telomerase inhibition via RNAi-mediated knock-down of \( hTERC \) and \( hTERT \) (Supplementary Fig. S4). In this experiment, cells were not expanded until the telomere-dependent senescence checkpoint.

**Cancer pathway profiling in derived tumors**

In order to characterize the transformation process from immortalized cells to premalignant cells and finally established tumors, we profiled various cancer pathways employing qPCR array technology. The analyses revealed the differential
expression of various cell cycle regulators involved in the execution of senescence, apoptosis, and DNA damage response programs. For instance, CDKN1A (p21) was downregulated in FH-hTERT pbcatS33Y and displayed a further decrease in FH-hTERT pbcatS33Y-derived tumors. In contrast, we observed an upregulation for CHEK2 (RAD53), RB1, the gene coding for the retinoblastoma protein, and the transcription factor E2F1, in FH-hTERT pbcatS33Y as well as in derived tumors. Expression of the tumor suppressor TP53 was not significantly altered in investigated tumor samples. However, S100 calcium binding protein A4 (S100A4), a strong plasminogen activator inhibitor, was upregulated by more than 2-log levels in 3 out of 4 assessed tumors (Fig. 5A). The integrins ITGA1, ITGA2, and ITGA3, were all decreased in derived tumors, whereas the integrin ITGA4 (also known as CD49d) was 10-fold upregulated in FH-hTERT pbactS33Y and in derived tumors (Fig. 5B). Furthermore, the matrix metalloproteinase 1 (MMP1) displayed elevated and the plasminogen activator inhibitor SERPINE1 decreased expression levels in FH-hTERT pbcatS33Y and in derived tumors. Urokinase-type plasminogen activator (PLAU) also plays a major role in cancer invasion and was induced by β-catenin activation in our model system with high expression in most investigated tumors (Fig. 5C). Interestingly, vascular endothelial growth factor A (VEGFA) was not elevated in the majority of derived tumors. However, we observed an upregulation of insulin-like growth factor (IGF) in samples with low VEGFA and a strong suppression of thrombospondin 1 (THBS1), a strong inhibitor of neovascularization and tumorigenesis (Fig. 5D).
Discussion

The development and progression of cancer is accompanied by complex changes in gene expression patterns and multiple genetic alterations. To this regard, genome-wide cDNA microarray studies have been successfully used to identify differences in gene expression between HCC and the surrounding non-malignant liver tissue (29, 30). Nevertheless, based on HCC molecular signatures, it is currently not possible to attribute functional (cancer driving) properties to individual genetic hits or expression clusters, and to define a sequential hierarchy for the identified molecular changes. In contrast to descriptive genome-wide studies evaluating clinical samples, our cell culture model offers the possibility to study functional consequences of distinct genetic alterations. We have previously demonstrated that telomerase activation confers immortality (unlimited replicative potential) without inducing a transformed phenotype (25). The current study provides, for the first time in an untransformed human model system, evidence that an aberrant activation of Wnt/β-catenin signaling accelerates proliferation with reduced serum-dependence. Therefore, β-catenin activation further promotes the transformation process and, by establishing self-sufficiency in growth signals and eliminating contact inhibition, increases the risk for malignant transformation. Moreover, these growth accelerating functional consequences of Wnt/β-catenin activation result in a permissive cellular phenotype characterized by sustained proliferation despite the acquisition (or possibly even promotion) of structural cytogenetic changes. In our study, cytogenetic monitoring revealed the development of persisting translocations in β-catenin activated cells, as shown by their clonal appearance. This has not been observed in telomerase-immortalized clones without forced β-catenin signalling during long-term expansion.
culture (25); and thus, indicates that additional mutation events are tolerated in telomerase and β-catenin positive human fetal hepatocytes and seem to be required for the establishment of a full cancer phenotype. As proof of principle for the driving role of β-catenin activation in hepatocarcinogenesis, only a few passages in cell culture resulted in a transformed phenotype with anchorage-independent growth and tumor formation in athymic nude mice.

Although only 20-30% of HCC samples exhibit a direct activation of Wnt/β-catenin signaling induced by mutations in the APC or CTNNB1, 70% of all cases show upregulation of the pathway (19). As additional support to the already known involvement of β-catenin signaling in proliferation and survival of tumor cells, it has recently been demonstrated that in the absence of NFκB activation, resistance towards apoptosis in premalignant hepatocytes was sustained by an aberrant β-catenin signaling (31). Clearly, animal models suggest that aberrant β-catenin signaling is by itself not sufficient to induce malignancy. To this regard, Harada and colleagues constructed a mouse strain (Catnb$^{lox(ex3)}$) containing a mutant β-catenin allele with exon 3 sandwiched by loxP sites. Cre-mediated deletion of β-catenin phosphorylation sites, resulting in pathway activation, caused numerous adenomatous polyps in the intestines but no neoplastic foci in the liver (32). In line with this finding, patients with APC mutations do not seem to be characterized by an increased frequency of HCC. Taken together, these and our observations corroborate that in addition to telomerase and β-catenin activation further oncogenic mutations are required to establish a full cancer phenotype. On the other hand, activation of β-catenin signaling provided an additional proliferative advantage during c-Myc/E2F1-driven hepatocarcinogenesis (33). This proliferative advantage of
immortalized cells with forced β-catenin pathway activation has been functionally confirmed for human liver cells in our model system. To this regard, RNAi experiments in our cell clones established that hTERT and telomerase activity are not required to maintain proliferation in transformed cells. It still needs to be clarified, how frequently immortalized cells with forced β-catenin activation escape telomere-dependent cell cycle arrest following telomerase inhibition.

Intriguingly, patients with HCC and β-catenin mutations frequently lack any significant fibrosis or cirrhosis (34). In addition recent publications report induction of Wnt/β-catenin signaling by the hepatitis B virus X protein (35) and the core protein of the hepatitis C virus (36) as potential mechanism driving hepatocarcinogenesis in a non-cirrhotic liver. These reports and our observation that β-catenin activation induces a cancer permissive phenotype in telomerase-immortalized cells, strongly support the notion that β-catenin dysregulation is one of the critical hits in the development of HCC. In the light of these findings, the proposed therapeutic application of β-catenin activation to promote progenitor cell-driven liver regeneration in hepatic failure or in small-for-size grafts following liver transplantation (34) should be explored with great caution.

In tumors derived form β-catenin-active telomerase-immortalized cells, cancer pathway profiling revealed upregulation of VEGFA or IGF1, MMP1, and PLAU. MMPs play an important role in cancer cell invasion by degrading extracellular matrices. For instance, HCC cells constitutively expressing MMPs can promote cells to invade through matrix gel in vitro and this MMP-dependent invasion is increased in response to hepatocyte growth factor and is blocked by MMP inhibitors (37).
Moreover, the plasminogen activator PLAU was activated by β-catenin in our model system. Interestingly, in all investigated tumors we also observed a 2-log level suppression of SERPINE1, one of the major plasminogen activator inhibitors. A recent analysis confirmed that PLAU downregulation leads to decreased migration and proliferation abilities of HCC cells (38). Interestingly, VEGFA was not upregulated in all investigated tumors. Alternatively, angiogenesis in our model system seems to be mediated by IGF1 and suppression of THBS1. To this regard, a screening of clinical samples revealed a correlation between THBS1 expression and tumor invasiveness and progression in HCC (39).

In summary, our results recapitulate multistep hepatocarcinogenesis driven by telomerase activation and Wnt/β-catenin signaling. Unlimited proliferation conferred by telomere stabilization is an early requirement during the transformation process; however, not sufficient to induce a malignant phenotype. Additional β-catenin activation induces cancer cell characteristics and further promotes the transition from immortalization to malignant transformation. Transformation in β-catenin positive hTERT-immortalized cells is associated with the acquisition of translocations, micro-amplifications, and deletions.

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and B. Otto (Array Service Center of the Department of Clinical Chemistry, University Medical Center Hamburg-Eppendorf).
References


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<td>Huh7 pbcatS33Y</td>
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NA, not applicable. Means ± SD. * P vs Huh7 < 0.05, *** P vs PD 35-40 < 0.001.
Table 2. Tumor formation in athymic nude mice

<table>
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<tr>
<th>Cell clone</th>
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<th>Tumors (%)</th>
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<td>FH-hTERT pbcatS33Y</td>
<td>16</td>
<td>16 (100)</td>
<td>19.0 ± 3.0***</td>
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<tr>
<td>Huh7</td>
<td>8</td>
<td>7 (87.5)</td>
<td>7.7 ± 2.6</td>
<td></td>
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<tr>
<td>Huh7 pbcatS33Y</td>
<td>10</td>
<td>9 (90)</td>
<td>3.4 ± 0.9***</td>
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NA, not applicable. Means ± SD. *** P vs Huh7 < 0.001.
FIGURE 1. Constitutive β-catenin activation. A, relative expression levels of β-catenin were determined by qPCR with a basket housekeeper as internal control and one FH-hTERT pcDNA sample as calibrator (expression level = 1). Bar graphs represent average expression levels (SD = error bars). B, accumulation of β-catenin was evaluated by immunoblot. HepG2 cells served as a positive control (typical additional truncated 75 kDa β-catenin band) and β-actin was visualized as a loading control. C, a dual-luciferase reporter assay was performed to determine CRT. HepG2 cells were assayed as a positive control. Bar graphs (SD = error bars) represent average CRT, expressed as luciferase activity ratio M50/M51 following correction for transfection efficiency.

FIGURE 2. Proliferation analyses. A, proliferation was monitored by serial cell counts at different time points after seeding. Cell counts were obtained from 4 wells per time point. B, for colorimetric measurements of cell density, 8 wells were analyzed per time point. Depicted are means ± SD (error bars). Activation of β-catenin signaling (FH-hTERT pbcatS33Y) led to a significantly enhanced proliferation (* P < 0.05, ** P < 0.01). C, to investigate serum dependence, cells were cultured with 0 or 2% serum. Cell density was determined 72 hours after seeding by colorimetric assay (OD vs 12 hours). Bar graphs (SD = error bars) represent average OD of 8 wells. D, to determine contact inhibition, cell cycle profiles of near-confluent (40-60%) and super-confluent (>100%) cultures were obtained by flow cytometry. Histogramms were analyzed by ModFit to attain S-phase fractions. Bar graphs show cell cycle distribution and calculated s-phase reduction (in % compared to near-confluent cultures).
FIGURE 3. Cytogenetic investigations. A, mFISH demonstrated clonal structural chromosomal aberrations in FH-hTERT pbcatS33Y, shown is a representative karyotype with the translocations t(5;19)(q31;p11orq11) and t(10;11)(p13;q21). B, SNP-arrays were performed to detect micro-amplifications (gains) and deletions (loss) in DNA samples obtained from FH-hTERT pbcatS33Y in comparison to the HapMap reference and FH-hTERT controls. New gains and losses in FH-hTERT pbcatS33Y are marked (red circle).

FIGURE 4. Tumor formation. A, FH-hTERT pbcatS33Y cells were transplanted subcutaneously into the dorsal flanks of athymic nude mice. Tumor volumes were measured three times per week using a caliper. The photographs show representative mice with tumor growth observed in all transplantation sites. B, tumor growth curves for transplanted Huh7 pbcatS33Y and FH-hTERT pbcatS33Y cells were generated from caliper measurements. Day 0 was defined as first day of detected tumor growth to adjust for different latency periods until tumor formation. Mice with a tumor volume > 0.8 cm³ were sacrificed.

FIGURE 5. Cancer pathway profiling. Expression studies were performed in FH-hTERT pbactS33Y and samples from 4 different tumors employing qPCR array technology profiling genes involved in tumorigenesis. Normalized expression levels were compared to FH-hTERT pcDNA as calibrator (expression level = 1). Assessed genes are part of biological pathways involved in, A, cell cycle control and DNA damage repair, B, adhesion, C, invasion and metastasis, and D, angiogenesis.
FIGURE 1

A

- FH-hTERT pcDNA
- FH-hTERT pbcatwt
- FH-hTERT pbcatS33Y

Relative expression CTNNB1

P < 0.01
P < 0.05

B

- kDa
- β-Catenin 92
- tβ-Catenin 75
- β-Actin 43

C

- HepG2
- FH-hTERT pcDNA
- FH-hTERT pbcatwt
- FH-hTERT pbcatS33Y

CRT (normalized to M50/M51)

P < 0.01
P < 0.05
FIGURE 2

A

![Graph A: Cell count over time](image)

B

![Graph B: OD vs time](image)

C

![Graph C: Increase in OD vs 12 h](image)

D

![Graph D: Cell cycle distribution and S-phase reduction](image)
FIGURE 3

A

B

Gain
Loss
FIGURE 4

A

B

FH-hTERT pbcatS33Y
Huh7 pbcatS33Y
FIGURE 5

A

B

C

D

Relative expression

FH-hTERT pcDNA

FH-hTERT pbcatS33Y

Tumor 1

Tumor 2

Tumor 3

Tumor 4
Molecular Cancer Research

Forced activation of β-catenin signaling supports the transformation of hTERT-immortalized human fetal hepatocytes

Henning Wege, Denise Heim, Marc Lütgethetmann, et al.

*Mol Cancer Res* Published OnlineFirst August 1, 2011.

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