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Human *telomerase reverse transcriptase* promoter is activated during liver regeneration and hepatocyte proliferation

Running title: Regulation of telomerase in liver cells

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Abstract

Background & Aims. Telomerase activity is non-detectable in healthy human liver biopsies but it is up-regulated in most human liver cancers. There is an ongoing debate whether telomerase is activated in response to acute or chronic liver injury. Telomerase activity is closely attributable to the expression of its catalytic subunit *telomerase reverse transcriptase* (*TERT*). Here we analyzed the activity of the human *TERT* (*hTERT*) promoter during liver regeneration *in vivo* and hepatocyte proliferation *in vitro*. Methods. As an *in vivo* model we used the hTERTp-*lacZ* transgenic mice, which contain an 8.0 kbp fragment of the *hTERT* gene promoter. Liver regeneration was induced by partial hepatectomy. As an *in vitro* model we used the HepaRG cell line as a new model system for human hepatocyte proliferation and differentiation. Results. *hTERT* promoter activity showed a significant increase after partial hepatectomy. The *hTERT* promoter activity is induced in hepatocytes as shown by immunohistological analysis. Similar to the *in vivo* results, telomerase activity and *hTERT* expression were up-regulated in proliferating HepaRG cells and repressed in response to growth arrest and differentiation. Moreover, we show that telomerase activity is essential for HepaRG proliferation. Promoter mapping revealed that a proximal 0.3 kbp fragment harbors all necessary elements for the regulation of *hTERT* gene in HepaRG cells. We further identified E2F2 and E2F7 as main factors governing the differential expression of *hTERT* gene in proliferating hepatocytes *in vitro* as well as *in vivo*. Conclusions. This is the first experimental evidence indicating that *hTERT* is induced in hepatocytes during liver regeneration. These data imply a pivotal functional role of telomerase activity in human liver.

Keywords: telomerase; liver regeneration; hepatocytes, E2F

Introduction

Telomerase is a reverse transcriptase that is required for the maintenance of chromosome ends and prevention of progressive loss of DNA during genome replication. In humans, telomerase is active during early embryogenesis but is repressed in most adult tissues. Exceptions are germ-line cells and stem cells of the intestinal epithelium, the basal layer of skin, and the hematopoietic system. In addition, reactivation of telomerase is observed during immortalization *in vitro*, and a marked up-regulation of telomerase activity occurs in 80% of all human tumors including liver cancer (for review see El-Serag & Rudolph, 2007).¹ Although the activation of telomerase during tumorigenesis and its contribution to the increased proliferation capacity of stem and progenitor cells is well established, less is known about its regulation in normal cellular growth and tissue homeostasis.

The liver is unique concerning its regeneration capacity. Quiescent hepatocytes proliferate after partial hepatectomy. Up to 75% of the liver can be removed and the remaining tissue can successfully regenerate the entire organ mass within a few weeks. However, the regenerative capacity of the liver is reduced in the elderly.^{2,3} In addition, it is known that the regenerative capacity of the liver declines at the end stage of chronic diseases, such as hepatitis. After long periods of times (20-40 years) the proliferative capacity declines coinciding with the evolution of cirrhosis.⁴ The decline in regenerative capacity and the evolution of cirrhosis in response to chronic liver injury in humans is associated with telomere shortening and hepatocyte senescence. Whether telomerase activity contributes to the long lasting regenerative activity of human liver cells remains unclear. The liver contains bipotent stem and progenitor cells.⁵ However, differentiated hepatocytes contribute to liver regeneration in

response to injury and labelling of regenerating liver cells in mice revealed that 80-90% of the liver cells participate in regeneration.⁶

Activation of telomerase could contribute to the maintenance of regenerative reserve in adult human liver. A weak induction of telomerase activity has been observed in patients with chronic liver diseases.^{7, 8} However, it remains a debate whether this activation truly reflects telomerase activity in hepatocytes. The fact that knockout mice lacking telomerase activity have reduced regeneration capacity indicates however that telomerase activity can contribute to liver regeneration.⁹ Recent reports indicated that expression of TERT can also exert some telomere independent effects that could be important for regulation of tissue regeneration in response to injury. It was shown the TERT acts as a co-factor controlling transcriptional regulation of Wnt target genes in mice.¹⁰

Previous reports on telomerase activity during experimental liver regeneration studies in mice and rat delivered inconsistent results.¹¹⁻¹³ A major problem in these studies is that rodents, in particular mice, show a more ubiquitous expression of telomerase as compared to humans. Telomerase activity is clearly detectable in mouse liver but not in resting human liver. The limiting component restricting telomerase activity in human tissues is the catalytic subunit of the telomerase TERT.¹⁴ Indeed, there is a marked difference in *TERT* mRNA levels in human and mouse livers.¹⁵ Together, these results indicate that there is an important, species-specific difference in the regulation of *TERT* during liver regeneration. There is experimental evidence that the differential regulation is based on different promoter organization.^{16, 17} Several transcription factors responsible for the regulation of the human and mouse *TERT* gene expression have been described, including c-myc, Sp1, CTCF and E2F factors, among others (for review see Kyo et al.).¹⁸ Cell- or tissue-specific regulation of telomerase may be

attributable to the differential occupation of the *hTERT* promoter by the trans-acting factors. To understand the regulation of telomerase activity in human tissues an analysis of human *TERT* promoter activity is required.

In this study, using the human *TERT* promoter (hTERTp)-*lacZ* transgenic mouse model^{15, 19} we provide the first experimental evidence that human *TERT* promoter is activated in hepatocytes during liver regeneration in response to acute liver injury as induced by partial hepatectomy as well as in proliferating human hepatocytes in culture using the HepaRG cell line, a new model system for human hepatocytes.²⁰ These studies support the new concept that activation of telomerase in adult hepatocytes contributes to the maintenance of liver regenerative capacity in humans. Furthermore, promoter mapping, *in vivo* and *in vitro* siRNA gene knock-down and chromatin immunoprecipitation studies revealed E2F2 and E2F7 as key activators of *hTERT* gene whereas *Rb* seems to be responsible for *hTERT* repression in liver cells.

MATERIALS AND METHODS

Cell culture, transfection and plasmids. HepaRG cells were cultivated as published.²⁰ The following *hTERT* promoter constructs driving luciferase expression were used to generate stable transfectants: pGRN310 (originally obtained from Geron Inc.), pGL2-297 (0.3 kbp *hTERT* promoter fragment) was derived from pGRN310. The *hTERT* promoter mutant constructs hm1 to hm14 were kindly provided by Dr. I. Horikawa. pBABE-Puro, pBABE-hTERT and pBABE-hTERT-DN were kindly provided by R. Weinberg. shRNA for *hTERT* (sh-*hTERT*) was received from Addgene. HepaRG cells were cotransfected with *hTERT*-promoter constructs (pGRN310 and pGL2-297) and a neo-selectable plasmid pCDNA3 using PEI (Polysciences). Cells were selected with G418 (800 ug/ml) in order obtain stable clones. Luciferase activity was determined using the Dual Luciferase Assay Kit (Promega). siRNA transfections were performed in triplicates in 12-well or 24-well plates by Lipofectamin RNAiMAX according to the recommendations of the supplier (Invitrogen). Mouse hepatocellular cancer cell line AL1698 was established in our laboratory from a liver specific TRP53 null C57BL/6J mouse (unpublished results).

Colony formation assay. HepaRG cells were plated at a concentration of 2×10^5 in 6-well plates. Cells were transfected with 8 μ g of indicated cDNAs and subcultured into 10 cm dishes 36 hrs later in medium containing puromycin (1 μ g/ml). Drug-resistant colonies appearing two weeks later were fixed with methanol, stained with Giemsa and counted.

SDS-PAGE and immunoblot analysis. For protein analysis cells were lysed with 4xLaemmli buffer, resolved by SDS-PAGE, and then transferred to nitrocellulose membranes as described previously.²¹

Chromatin immunoprecipitation. ChIP experiments were performed as described by Weinmann et al.²². Briefly, ~1x10⁷ HepaRG cells or liver samples (~1g pooled from 2-3 livers) were fixed with 1% formaldehyde and sonicated chromatin was incubated at 4°C overnight with the antibodies against E2F2 (sc-633), E2F7 (sc-32574), Rb (G3-245, BD Pharmingen) and pre-immune serum, respectively. Immunocomplexes were then incubated with protein A/G agarose beads, washed, eluted from the beads, cross-linked, and harvested. After purification, the DNA fragments were analyzed by PCR using iTaq SYBR Green supermix with Rox (Biorad). PCR conditions were as follows: 1 cycle at 94 °C for 5 min; 36 cycles at 94 °C for 15 s and at 60°C for 1 min.

Mice. Male *hTERTp-lacZ* and wild-type C57/B6J mice (age 8–10 weeks) were used for this study. Mice were kept in SPF (special pathogen free) conditions in the laboratories of Heinrich-Pette-Institute, Hamburg. Two-thirds PH was performed by the method of Higgins and Anderson.²³ Mice were anesthetized and subjected to 70% PH as described earlier.⁶ Following PH, groups of four mice were sacrificed at 0, 24, 48, 72, 96 and 120 h and the livers were removed and immediately frozen in liquid nitrogen or formalin fixed and paraffin embedded.

High-pressure, high-volume tail vein injections of plasmid DNA or siRNA. The tail vein injections were done as described by Zhang et al.²⁴ for plasmid DNA and by Zender et al.²⁵ for siRNA. Briefly, 100 µg of *hTERTp-luciferase* reporter plasmids (WT, hm1 and hm4) per mouse or ~15 nmol of siRNA per mouse (0.5 nmol per gram of body weight) were resuspended in 2.5 ml Ringer's solution (147 mM NaCl, 4 mM KCl, 1.13 mM CaCl₂) and injected over a period of 5-10 seconds. In injection experiments, PH (five mice each group) was performed 48 hours after plasmid DNA injections or 24 hours after siRNA injections.

Beta-Galactosidase activity. Beta-Galactosidase activity was measured with Enhanced β -Galactosidase Assay Kit (CPRG) according to the recommendations of the suppliers (Genlantis).

RNA isolation, radioactive RT-PCR. 20-50 mg tissue was homogenized on ice in peqGOLD RNA PURE solution (Pepylab) with FastPrep Homogenisator FP120 (Q-Biogene) and RNA was prepared according to the recommendations of supplier. Semi-quantitative and radioactive RT-PCR reaction were performed as described earlier.¹⁵

Quantitative real-time PCR. Quantitative real-time PCR was performed with AB7300 (Applied Biosystems). For the reverse transcription (RT) step, cDNA was synthesized from 500ng of total RNA using the SuperscriptIII kit (Invitrogen). The RT reaction was diluted to 1/10 and 2 μ l of the diluted cDNA was used for the quantitative PCR (qPCR) in a total volume of 10 μ l using iTaq SYBR Green supermix with Rox. At least three biological and three technical replicates were done for quantification.

Extract preparation and TRAP assay. The endogenous enzymatic activity of telomerase in mouse liver and HepaRG cell extracts was determined by Telomeric Repeat Amplification Protocol (TRAP) with the TRAPeze kit (Serologicals Corporation) as described earlier.¹⁵

Immunohistochemistry. Tissues were routinely fixed in 4% buffered formalin and paraffin-embedded. After deparaffinization, β -galactosidase immunostaining was performed using the ChemMate™Envision™ Detection Kit (DakoCytomation) as described earlier¹⁵ using purified mouse anti-*Escherichia coli* β -galactosidase monoclonal antibody (Promega) at a

concentration of 4 µg/ml. Tissue sections were counterstained by hematoxylin and permanently mounted.

Statistical Analysis. The Mann-Whitney U-test and the t-test were used to calculate the statistical significance and standard deviations by using Graphpad Prism and Microsoft Excel softwares.

Results

Human *TERT* promoter activity is up-regulated in regenerating liver

We have previously shown that the transgenic expression of the *lacZ* reporter gene driven by an 8.0 kbp promoter fragment of the *hTERT* gene reflects the expression pattern of the *hTERT* gene in normal and malignant tissues.¹⁵ Specifically, *hTERT* promoter activity was seen in stem cell compartments of the testis and in tumor cells, but not in normal somatic tissues. To investigate *hTERT* promoter activity in liver cells *in vivo* we determined *hTERT* promoter activity during regeneration induced by two-thirds partial hepatectomy (PH). mRNA levels of the *lacZ* reporter gene and endogenous *mTert* gene were analyzed by radioactive RT-PCR and by quantitative real-time RT-PCR. We observed a marked induction of the *lacZ* mRNA at day 3 after partial hepatectomy (Fig. 1A, B). In line with the RNA data, a 3-5-fold increase in β -galactosidase (β -Gal) enzymatic activity was detected, starting at day 3 after PH (Fig. 1C). In contrast, endogenous mouse *Tert* (*mTert*) was constitutively expressed and only showed a weak increase in response to PH (Fig. 1D). Likewise, telomerase activity was constantly detectable in mouse liver and was not significantly induced by PH (Fig. 1E).

To determine cell-type specific expression of the β -Gal reporter protein we performed immunohistochemistry on liver sections (Fig. 1F). No detectable β -Gal signal was observed in resting liver (day 0) of *hTERTp-lacZ* transgenic mice (Fig. 1Fb). On the contrary, β -Gal protein was detectable in the majority of hepatocytes at day 5 after PH (Fig. 1Fd). Liver sections from wild-type mice served as controls where no background signals were detected (Fig. 1Fa, Fc).

Telomerase activity is required for proliferation and is repressed during HepaRG differentiation

To analyze the functional role of telomerase for hepatocyte proliferation and to elucidate the molecular mechanisms underlying *hTERT* gene regulation in liver cells we used the recently established HepaRG cell culture model. In the differentiated (non-proliferating) state, these cells exhibit features of primary human hepatocytes.²⁰ When seeded at low density (1×10^5 cells per well into 12-well plates), HepaRG cells proliferate up to 5-6 days post-plating ceasing proliferation from thereon (Fig. 2A) accompanied by the increase of hepatocyte-specific markers *pre-albumin* and *APOA1* during the differentiation of HepaRG cells (Fig. 2B). The increase of *pre-albumin* mRNA preceded an increase in pre-albumin protein amounts as determined by immunoblot experiments (Fig. 2C) and the appearance of cells exhibiting a hepatocyte-like morphology (Fig. 2D). Telomerase activity was determined at indicated time-points after induction of differentiation (Fig. 3). No significant change in telomerase activity was observed during the first five days post-plating followed by steady decrease during continued culturing. At day 14, only a weak telomerase activity could be detected which completely disappeared after DMSO-treatment for 14 days from hereon (Fig. 3A). Together, these data indicated that suppression of telomerase activity correlated well with growth arrest and differentiation of HepaRG cells. The level of telomerase activity in human cells and tissues is tightly linked to the expression level of *TERT* gene. A significant decrease in *hTERT* mRNA amounts (to ~ 40% of the initial mRNA levels) was detectable as early as day 2 post-plating and continued to decrease to basal/non-detectable levels at around day 5 post-plating (Fig. 3B). This down-regulation is not due to an exhaustion of extrinsic supplements as the medium was replaced every 2nd day during the complete culturing period.

This result let us assume that the down-regulation of telomerase activity is due to an intrinsic regulatory mechanism coupled to decrease in cell proliferation.

We then asked whether telomerase activity was required for the proliferation of HepaRG cells and functionally knocked-down endogenous telomerase activity either by the over-expression of a DN-*hTERT* or by an shRNA directed against *hTERT* mRNA (Fig. 3C, D). Over-expression of DN-*hTERT* strongly reduced cell growth as determined by colony formation assay (Fig. 3E) whereas the over-expression of wild-type *hTERT* resulted in constitutive activity of telomerase (Fig. 3C) and in a significant increase in the number of colonies (Fig. 3E). Similar results were obtained by shRNA mediated knock-down of *hTERT* gene expression and telomerase activity (Fig. 3D, E).

Defining regulatory elements controlling *hTERT* expression in human hepatocytes

In an attempt to define the regulatory region of *hTERT* promoter we generated HepaRG cells stably carrying luciferase reporter vectors (i) under the control of an 8 kbp (corresponding to the 8 kbp *hTERT*p-*lacZ* construct in the transgenic mice) and (ii) under the control of an 0.3 kbp core promoter directly upstream of the transcription start site (Fig 4A). Parental HepaRG cells, control clones and *TERT* promoter clones were treated in the same way as described above and luciferase activity was determined (Fig. 4B). Both promoters were down-regulated with similar kinetics indicating that the regulatory elements that are responsible for *hTERT* gene expression must reside within the short promoter fragment. Further, the kinetics of *hTERT* promoter reporter activity was similar to the down-regulation of the endogenous *hTERT* mRNA. A slight difference in decay of luciferase activity and *hTERT* mRNA levels

might be due to different stability of luciferase mRNA (half-life ~4-10h in human cells) and *hTERT* mRNA (half-life ~1h in human cells).²⁶⁻²⁸

As the 0.3 kbp fragment was repressed during HepaRG cell differentiation we aimed at identifying the regulatory sequences which might be responsible for *hTERT* promoter activity in hepatocyte proliferation. For this purpose we used *hTERT* core promoter constructs with mutations (Fig. 4C), which destroy the putative transcription factor binding sites within this 0.3 kbp core promoter.¹⁷ Plasmids were transfected in HepaRG cells and luciferase activity was determined. Disruption of single E-box (hm2 or hm14), GC-box (hm3, hm5), and E2F factor (hm1) binding sites results in approximately 40-50% reduction of *hTERT* promoter activity. Mutation of another E2F factor binding site (hm4) and of some putative CTCF-binding sites (hm8, hm10) resulted in low but significant reduction of *hTERT* promoter activity. Interestingly, mutation of another CTCF binding site (hm6) resulted in a two-fold increase of *TERT* promoter activity compared to the construct with wild type *hTERT* promoter sequence (Fig. 4D).

Expression levels of E2F2 and E2F7 factors correlate best with *hTERT* promoter activity both in HepaRG cells and during liver regeneration

We have determined expression levels of several factors in HepaRG cells and in regenerating mouse liver. In HepaRG cells, mRNA levels of *E2F1*, *E2F2*, *E2F7* and *E2F8* showed similar kinetics during differentiation (Fig. 5A) whereas other E2F factors (*E2F3*, *E2F4*, *E2F5* and *E2F6*) were either not detectable or did not show any change in mRNA levels during HepaRG differentiation (not shown). Similarly, we could not observe any difference in both mRNA and protein level of Sp1 (a GC-box binding protein), c-myc and CTCF genes (data not

shown). The kinetics of *E2F1*, *E2F2*, *E2F7* and *E2F8* expression resembled that of the endogenous *hTERT* during the course of HepaRG differentiation. Next, we determined mRNA levels of these factors in liver samples (Fig. 5B). Interestingly, only mRNA levels of *E2F2* and *E2F7* showed a close correlation to *lacZ* mRNA levels (i.e. *hTERT* promoter activity) whereas expression of *E2F1* and *E2F8* very closely correlated with the peak of DNA synthesis at day 2 after PH. Again no change in other E2Fs (*E2F3*-*E2F6*), *Sp1*, *c-myc* and *CTCF* mRNA levels was detectable (not shown). We also determined changes in protein amounts for E2F2 and E2F7 in HepaRG cells and liver samples. Proteins amounts correlated well with RNA amounts during differentiation of HepaRG cells (Fig. 5C) and during liver regeneration (Fig. 5D). In liver, E2F2 and E2F7 protein amounts accumulate from day 3 on and show strongest signals at day 5 after PH whereas *hTERT* promoter activity is reduced after day 4 after PH. This result indicates that both factors may regulate additional genes at later time-points during liver regeneration.

E2F2 and E2F7 regulate *hTERT* promoter in HepaRG cells and in regenerating mouse liver

To elucidate the contribution of the suspected transcription factors on *hTERT* promoter activity, siRNA knock-down experiments were conducted in HepaRG cells that stably contained the 0.3 kbp *hTERT* promoter construct (HepaRG-297) (Fig. 6). In addition, a siRNA against luciferase gene was used as a positive control (siLuc). The siRNA transfection efficiency of HepaRG cells and the knock-down efficiency of the siRNAs were first determined (Suppl. Fig. 1). Using a red-labelled control siRNA the transfection efficiency of siRNAs was shown to be 80-100% in HepaRG cells (Suppl. Fig. 1G). In case of *E2F2* and *E2F7* genes a set of 4 different siRNAs was tested, either separately or in combination (Suppl.

Figs. 1E, 1F). The most efficient *E2F2* and *E2F7* single siRNAs were then used for the following knock-down experiments. Transfection of proliferating HepaRG-297 cells with siLuc resulted in reduction of luciferase activity to nearly 20% (Fig. 6A) indicating that the experimental set-up was accurate. We also observed a significant reduction of *hTERT* promoter activity if siRNAs directed against *E2F2*, *E2F7* and *CTCF* were used (Fig. 6A). Luciferase activity, i.e. *hTERT*-promoter activity was most significantly reduced with *E2F7* siRNA in proliferating cells.

To determine their impact on endogenous *hTERT* mRNA levels we measured the expression levels *hTERT* mRNA in cells transfected with *E2F*- and *CTCF*-siRNAs (Fig. 6B). Down-regulation of *E2F7* had the most significant impact on *hTERT* mRNA levels (~4-fold reduction compared to control siRNA transfected cells). In comparison to control siRNA transfected cells, *hTERT* mRNA levels were about the half in *E2F2*-siRNA transfected cells whereas *E2F1*, *E2F8* and *CTCF* knock-down resulted only in mild reduction of *hTERT* mRNA levels (10%, 16% and 20% reduction in comparison to control siRNA transfected cells, respectively). The knockdown of *E2F7* appeared to be slightly more efficient in preventing *TERT* activation compared to *E2F2* knockdown. The combined knock-downs of *E2F2* and *E2F7* resulted in a similar reduction of *hTERT* mRNA levels compared to *E2F7* knock-down alone. Together, these results indicate that both *E2F2* and *E2F7* contribute to *hTERT* promoter regulation in hepatocyte cell culture. Of note, the prolonged down-regulation of *E2F2* or *E2F7* by shRNA impaired the proliferation capacity of HepaRG cells (Suppl. Figs. 2 and 3). This is an interesting observation as *E2F2* or *E2F7* null mice are fully viable.

To analyze whether *E2F2* and *E2F7* regulate *hTERT* promoter activity in the regenerating liver *in vivo*, we performed two types of experiments. Firstly, we injected the 0.3 kbp wild-

type or E2F-site mutant *hTERT-Luc* reporter plasmids (hm1 and hm4: the same that were used in the HepaRG cells in Fig. 4) in wild-type mice and measured luciferase activity in resting liver and after PH. Wild-type promoter activity exhibited a 2-3-fold increase at day 4 after PH compared to resting liver (both samples were collected at day 6 after plasmid injection). In contrast, the hm1 and hm4 mutant *TERT* promoters did not show an increase in reporter activity in response to PH (Fig. 6F). Secondly, we knocked-down endogenous *E2F2* or *E2F7* by high-pressure, high-volume mediated delivery of siRNA into the mouse liver and determined *hTERT* promoter activity by real-time RT-PCR quantitation of *lacZ* reporter gene (Fig. 6D) or β -Gal enzyme activity (Fig. 6E) in *hTERTp-lacZ* transgenic mice. The knock-down efficiencies of the siRNAs were determined in a mouse liver cancer cell line (Suppl. Fig. 4) and in liver samples (Suppl. Fig. 5). Of note, down-regulation of *E2F2* or *E2F7* prevented the induction of *hTERT* promoter activity in regenerating liver after PH (Figure 6E,F). Together, these experiments demonstrated that E2F factors regulate *hTERT* promoter activity during *in vivo* liver regeneration, specifically involving *E2F2* and *E2F7*.

E2F-Rb complexes bind to *hTERT* promoter

Binding of E2F-Rb complexes to *hTERT* promoter was described previously.²⁹ To analyze the association of E2F-Rb factors with *hTERT* promoter in human hepatocytes, ChIP experiments were performed (Fig. 7). We used HepaRG cells in proliferative (d1 post-plating) or differentiated state (d28 post-plating and DMSO-treated) (Fig. 7A). In addition, binding of the E2F factors to the *hTERT* promoter was analysed in liver samples of transgenic mice at d0 or at d3.5 after PH (Fig. 7B). Both *E2F2* and *E2F7* were found in complex with *hTERT* promoter. In contrast, Rb is associated with *hTERT* promoter in quiescent liver and in differentiated (non-proliferating) HepaRG cells but this binding was strongly reduced in

proliferating hepatocytes or in regenerating liver. Together, these results indicate a proliferation-dependent, dynamic occupation of the *hTERT* promoter by E2F-Rb factors in hepatocytes.

DISCUSSION

The current study provides the first experimental evidence that the expression of the human *TERT* gene is transcriptionally activated by E2F family proteins E2F2 and E2F7 in regenerating hepatocytes in response to liver injury *in vivo*. There was a continuous debate whether *hTERT* is activated in hepatocytes during liver regeneration. An activation of *TERT* transcription has been detected by *in situ* hybridization in large and dysplastic regenerative nodules in human cirrhosis.³⁰ However, its regulation in normal liver regeneration is still unclear. Telomerase activity is not detectable in human liver but some studies have reported a slight activation of telomerase in human liver during viral hepatitis^{31, 32} and lymphocytic infiltrates are a hallmark of chronic hepatitis. Indeed, telomerase activity can be induced in human lymphocytes.³³ Thus, the activation of *TERT* in chronic hepatitis was thought to reflect an activation of telomerase in infiltrating lymphocytes.³⁴

Experimental systems to investigate the activation of *hTERT* in regenerating hepatocytes have been missing. In contrast to humans, a constitutive expression of telomerase activity has been documented in mouse liver¹⁵ and this species-dependent regulation of *TERT* gene has been attributed to its promoter organization.^{16, 17} Here, a humanized mouse model was employed allowing to monitor *hTERT* promoter activity *in vivo* during liver regeneration. In a previous study we have shown that these mice show a human-like expression of *TERT* promoter activity in stem cells but undetectable activity in most somatic compartments.¹⁵ Using this experimental system, the current study demonstrates that the *hTERT* promoter is not active in resting liver but is markedly reactivated during liver regeneration. The expression of *hTERT* promoter activity was detectable in hepatocytes in regenerating mouse liver providing direct

evidence that hepatocytes have endogenous regulatory mechanisms that can reactivate telomerase.

In addition, using HepaRG cells as an *in vitro* model system for hepatocyte proliferation and differentiation our study shows that human hepatocytes also retain the intrinsic capacity to up-regulate *TERT* transcription and telomerase activity during proliferation. These results are in agreement with the *in vivo* data on the activity of the *hTERT* promoter in the regenerating liver of hTERTp-*lacZ* transgenic mice. These data together suggest that *hTERT* expression can be transactivated and suppressed in hepatocytes, both *in vivo* and *in vitro* during proliferation and quiescence, respectively. The current results indicate that the proliferation-dependent *hTERT* activation in hepatocytes involves E2F2 and E2F7 transcription factors. E2F-Rb factors have already been shown to regulate *hTERT* gene in different cell types.^{35, 29} Importantly, E2F1, E2F3 and E2F4 were shown to bind *hTERT* promoter in normal human fibroblasts but in this cell type E2F2 failed to bind *hTERT* promoter.²⁹ E2F7 has not been analyzed so far in the context of *hTERT* regulation. Our results indicate that E2F2 and E2F7 are the major activators of *hTERT* promoter in hepatocytes in response to proliferation whereas Rb is involved in *hTERT* repression in quiescent hepatocytes. Interestingly, binding sites for E2F-factors are not found in mouse promoter and may therefore account for this species-specific regulation of *TERT* gene expression. These results are in line with previous observations that the species-specific *TERT* gene expression is primarily due the differences of cis-acting sequences in the respective *TERT* gene promoters. The current study is also the first report on differential regulation of all known E2F factors during liver regeneration and liver cell differentiation. In particular, little is known about the physiological role of the recently identified E2F7 factor.^{36, 37}

Taken together, the current study provides the first direct evidence that *hTERT* expression is induced in proliferating hepatocytes *in vivo* and *in vitro*. *hTERT* could be one of the essential factors ensuring the regenerative capacity of human liver. According to this assumption, *hTERT* mutations could lead to an accelerated exhaustion of hepatocyte regenerative reserve during chronic liver disease. Interestingly, hepatic syndromes have been associated with *hTERT* mutations in patients with dyskeratosis congenita.³⁸ Moreover, two independent studies have revealed experimental evidence that *hTERT* mutations occur in 3-6% of cirrhosis patients with chronic liver disease (Hartmann et al. and Callado et al., Hepatology in press).

These findings support a new concept that activation of telomerase in adult hepatocytes contributes to the maintenance of liver regenerative capacity in humans. In principle, two mechanisms could be envisaged: (i) *hTERT* activation could stabilize telomeres in regenerating hepatocytes thus ensuring the high regenerative capacity of human hepatocytes. It is known that telomere shortening associates with end-stage chronic liver disease and evolution of cirrhosis.³⁹⁻⁴¹ These findings indicate that *hTERT* activity in human hepatocytes is not sufficient to completely maintain telomeres at a stable length in chronically damaged liver. These data stand in line with the observation that human hematopoietic stem cells express low levels of telomerase but yet experience age-dependent telomere shortening. Nevertheless, the activation of *hTERT* in human hepatocytes as shown in this study could be essential for the regenerative capacity of hepatocytes. (ii) *hTERT* activation could stimulate hepatocyte proliferation by acting as a transcriptional co-activator of Wnt signaling. The Wnt pathway is involved in tissue morphogenesis and is activated in various stem cell types and cancers. In a recent paper⁴² it was demonstrated that this pathway is activated in adult liver stem cells after liver damage. Recent studies have provided a proof of concept that TERT can act as a transcriptional co-factor in the Wnt-signaling pathway.¹⁰ It is conceivable that TERT

has similar functions in stem cells and somatic compartments. According to this hypothesis, the activation of *hTERT* in human hepatocytes could directly be involved in regulating Wnt target genes that are required for hepatocyte proliferation and liver regeneration. A more detailed analysis in further studies is required to elucidate a potential connection between Wnt signaling pathway and telomerase function during liver regeneration.

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Figure legends:

Figure 1. Up-regulation of *hTERT* promoter activity in regenerating mouse liver. (A) Radioactive RT-PCR for *lacZ*, *mTert* and as loading control, *gapdh*. (B) Real-time quantitative RT-PCR for *lacZ* and (C) determination of β -Gal enzymatic activity. (D) Real-time quantitative RT-PCR for *mTert* and (E) endogenous telomerase activity. (F) Immunohistological detection of β -Gal reporter protein in regenerating mouse liver. Immunofluorescence was performed on 4 μ m thick paraffin sections from livers of wild-type (a, b) and hTERTp-*lacZ* transgenic (c, d) animals as described earlier.¹⁵ Histology samples were visualized using a Zeiss Axiophot microscope and images were captured at 400x magnification using AxioCam MRc camera and AxioVision Version 4.7 imaging software (Carl Zeiss). Black arrows indicate hepatocytes without and red arrows indicate hepatocytes with hTERT promoter activation (β -Gal reporter protein expression).

Figure 2. Characterisation of HepaRG differentiation. (A) Determination of HepaRG proliferation. Cells were seeded at a density of 1×10^5 and cell numbers were counted at the indicated days post-plating in duplicate. (B) Determination of mRNA levels of liver specific differentiation markers *pre-albumin* (*transthyretin*) and *APOA1* (*Apolipoprotein A1*) by quantitative real-time RT-PCR. (C) Accumulation of liver specific protein pre-albumin by immunoblot. A significant increase in pre-albumin protein amount was observed in DMSO treated cells at day 28 post-plating, as a result of hepatocyte differentiation. (D) Morphology of HepaRG cells. Phase contrast micrographs of HepaRG cells under proliferating (2d pp) or differentiated (maintained for 14 days without DMSO, then treated with 2% DMSO for 14 days (28d pp) state. Cells were visualized using Olympus Mikroskop, and images were

captured at 100x magnification using Till Vision Software. Hepatocyte-like cells (arrow) and epithelium-like cells are indicated, respectively, by 'h' and 'e'.

Figure 3. Telomerase activity is required for HepaRG cell proliferation. (A) Radioactive TRAP assay. 200ng HepaRG cellular extract and an equivalent of 1000 cells from HeLa control cell extract were used for the TRAP assay. HeLa Δ , Heat treated HeLa cell extracts. CHAPS, TRAP assay without cellular extracts added. TSR8, TRAP control reaction according to the supplier's protocol. (B) *hTERT* gene mRNA level is down-regulated during HepaRG differentiation. Endogenous *hTERT* mRNA levels were determined by quantitative real-time RT-PCR. (C, D) Suppression of telomerase activity and reduction in *hTERT* mRNA levels in HepaRG cells. Cells were transfected with a vector encoding for a DN-hTERT protein or with an shRNA-sequence directed against *hTERT* mRNA and arising clones were used for TRAP assay (C) or *hTERT* mRNA determination (D). (E) Colony formation assay. HepaRG cells were transfected with empty vector (pBABE), wild-type hTERT (pBABE-wt-hTERT), pBABE-shTERT and pBABE-DN-hTERT and numbers of colonies were determined.

Figure 4. Core *hTERT* promoter activity is sufficient for *hTERT* promoter down-regulation during HepaRG differentiation. (A) Schematic drawing of 8.0 kbp and 0.3 kbp *hTERT* promoter luciferase reporter constructs. (B) Down-regulation of hTERTp-*Luc* reporter constructs in stably transfected HepaRG cells during differentiation was determined by luciferase assay. Relative luciferase activity was determined per mg protein extract. (C) Identification of cis-regulatory elements. Schematic drawing of 0.3 kbp *hTERT* promoter luciferase constructs with mutations (top); hm1-hm14 designate the single mutations.¹⁷ (D) Activity of mutant *hTERT* promoter constructs in proliferating or non-proliferating HepaRG

cells. HepaRG cells were transfected with reporter plasmids 1 day post-plating and luciferase activity was determined 2 days after transfection.

Figure 5. Determination of endogenous *E2F1*, *E2F2*, *E2F7* and *E2F8* mRNA levels by quantitative real-time RT-PCR (A) during differentiation of HepaRG cells (B) or in regenerating mouse liver. Determination of endogenous *E2F2* and *E2F7* protein levels by immunoblot (C) during differentiation of HepaRG cells (D) or in regenerating mouse liver.

Figure 6. *E2F2* and *E2F7* are potential regulators of *hTERT* in HepaRG cells and in regenerating mouse liver. (A) HepaRG-297 cells stably containing the core *hTERT* promoter were transfected with siRNAs directed against the indicated genes and luciferase activity was determined. Cells were transfected at 1 day post-plating and collected 2-3 days after transfection at ~30-50% confluency. Each experiment was performed at least three times in triplicates in 24-well plates. (B) Endogenous *hTERT* mRNA levels in HepaRG-297 cells transfected with *E2F*- or CTCF-siRNAs. The values are given in % mRNA levels compared to *hTERT* mRNA levels of untransfected control HepaRG cells (set to 100%). (C, D) siRNA mediated down-regulation of *E2F2* or *E2F7* impairs induction of the *hTERT* promoter activity during liver regeneration in *hTERTp-lacZ* transgenic mice as determined by *lacZ* mRNA levels (C) or β -Gal enzymatic activity (D). Wild-type but not *E2F*-site mutant (hm1 and hm4) *hTERTp-Luc* reporter is induced during liver regeneration (E). siRNAs or luciferase reporter plasmids were injected by high-pressure, large volume tail vein injection. Following injections, partial hepatectomy (five mice each group) was performed 48 hours after plasmid DNA injections or 24 hours after siRNA injections. Resected liver samples served as day 0 controls. Mice were sacrificed at day 4 (plasmid injections) or at day 3.5 (siRNA injections)

after PH for RNA and protein isolations. Luciferase activity was calculated relative to the activity of quiescent liver (0 hrs, set to 100%).

Figure 7. *In vivo* binding of E2F-Rb factors to *hTERT* gene promoter. Binding of endogenous E2F2, E2F7 or Rb factors on endogenous *hTERT* promoter was analyzed by ChIP assay in HepaRG cells (**A**) and in liver (**B**). E2F2 and E2F7 bind *hTERT* promoter in proliferating HepaRG cells (**A**) and in proliferating liver samples from *hTERT*p-*lacZ* mice (d3.5 after PH, n= 5 mice). (**B**) In contrast to the results on E2F2 and E2F7, Rb is associated with *hTERT* promoter in differentiated (non-proliferating) HepaRG cells (d28 pp) and in quiescent livers (0 hrs). At least three independent PCR reactions were performed for each sample.

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D

d2 pp

d28 pp

A

B

C

D

E

B

A

B

C

D

A **B** **C** **D** **E** **F** **G**

control sh-RNA

E2F2 sh-RNA

E2F7 sh-RNA

A

B