

Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis

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ABSTRACT Telomere shortening limits the number of cell divisions of primary human cells and might affect the regenerative capacity of organ systems during aging and chronic disease. To test whether the telomere hypothesis applies to human cirrhosis, the telomere length was monitored in cirrhosis induced by a broad variety of different etiologies. Telomeres were significantly shorter in cirrhosis compared with noncirrhotic samples independent of the primary etiology and independent of the age of the patients. Quantitative fluorescence in situ hybridization showed that telomere shortening was restricted to hepatocytes whereas lymphocytes and stellate cells in areas of fibrosis had significantly longer telomere reserves. Hepatocyte-specific telomere shortening correlated with senescence-associated β -galactosidase staining in 84% of the cirrhosis samples, specifically in hepatocytes, but not in stellate cells or lymphocytes. Hepatocyte telomere shortening and senescence correlated with progression of fibrosis in cirrhosis samples. This study demonstrates for the first time that cell type-specific telomere shortening and senescence are linked to progression of human cirrhosis. These findings give a novel explanation for the pathophysiology of cirrhosis, indicating that fibrotic scarring at the cirrhosis stage is a consequence of hepatocyte telomere shortening and senescence. The data imply that future therapies aiming to restore regenerative capacity during aging and chronic diseases will have to ensure efficient targeting of specific cell types within the affected organs.—Wiemann, S. U., Satyanarayana, A., Tсахuridu, M., Tillmann, H. L., Zender, L., Klempnauer, J., Flemming, P., Franco, S., Blasco, M. A., Manns, M. P., Rudolph, K. L. Hepatocyte telomere shortening and senescence are general markers of human liver cirrhosis. *FASEB J.* 16, 935–942 (2002)

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IN HUMANS, CIRRHOSIS is induced by a broad variety of hepatotoxins. Regardless of its etiology, cirrhosis evolves

slowly over many years, and chronic hepatocyte death and renewal are major predisposing factors (1–3). Although many studies have addressed the common end points of cirrhosis, little is known about the molecular lesions governing the progressive induction of cirrhosis during its long latency. Classical explanations propose that long-standing organ architectural changes induced by processes such as chronic inflammation, cytokine production, extracellular matrix reorganization, among others, become irreversible at some undefined point (1–3). Another not mutually exclusive thesis has proposed that sustained cellular turnover in chronic liver disease precipitates cellular senescence and/or crisis as a result of telomere shortening (4).

Telomeres are specialized nucleoprotein structures at the end of eukaryotic chromosomes (5). Continuous shortening of telomeres during each cell division limits the life span of primary human cells in vitro (6). It is still a matter of debate to what extent telomere shortening affects organo-pathophysiology during aging (7) or chronic diseases that induce elevated rates of cell turnover (8–11). The telomere hypothesis of liver cirrhosis proposes that chronic liver injury induces continual waves of liver destruction and regeneration, resulting in critical telomere shortening, which in turn culminates in hepatocyte replicative senescence or death and ultimately in liver cirrhosis (4). Experimental merit for the telomere hypothesis of cirrhosis comes from studies in the telomerase-deficient mouse (mTERC^{-/-}) showing defects in liver regeneration and a premature onset of cirrhosis in mice with short telomeres (12). In humans, a variety of studies from Japan described shortening of telomere restriction fragments (TRFs) in cirrhosis induced by viral hepatitis in patients over 45 years (11, 13–15). Nevertheless, it remains an open question whether the shortening of telomeres is a consequence of continuous liver regen-

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eration or a mechanistic factor triggering the development of cirrhosis.

To prove that telomere shortening plays a mechanistic role in human cirrhosis, it is necessary to show that telomere shortening is a general marker of cirrhosis independent of the etiology and patient's age. Since the cellular composition of the liver changes significantly at the cirrhotic stage (formation of fibrotic septa and lymphocyte infiltration), a cell type-specific analysis of telomere length is another step in understanding the role of telomere shortening in cirrhosis. We have analyzed telomere length in 76 cirrhosis samples induced by a broad variety of liver diseases (viral hepatitis, autoimmune hepatitis, alcoholism, primary sclerosing cholangitis, and primary biliary cirrhosis) in patients spanning a broad age range. A cell type-specific analysis of the telomere length was conducted on hepatocytes, fibroblasts, and lymphocytes using interphase Q-FISH, and the prevalence of senescence in cirrhosis was followed using senescence-associated β -galactosidase (β -Gal) staining. Our studies demonstrate that telomere shortening is a disease and age-independent sign of human cirrhosis and that telomere shortening and senescence are specifically found in hepatocytes but not in other cell types in the cirrhotic liver. Hepatocyte telomere shortening and senescence correlate with progression of cirrhosis. Our data strongly support the telomere hypothesis of human cirrhosis, indicating that hepatocellular telomere shortening and senescence represent a molecular mechanism in the evolution of human cirrhosis.

MATERIALS AND METHODS

Liver samples and histology

All liver samples were snap frozen in liquid nitrogen within 30 min after explantation and stored at -80°C .

Histological analysis was performed on formalin-fixed tissue samples at the Department of Pathology at the Medical School Hannover. The grade of inflammatory infiltration was scored on 52 cirrhosis samples by 2 investigators according to the Ishak criteria (16) (low grade, 18; moderate grade, 23; high grade, 11). The Child-Pugh criteria were used to classify disease progression in 72 samples according to standard criteria evaluating serum albumin, bilirubin, the grade of encephalopathy, ascites, and the prothrombin time (12 Child A cirrhosis, 32 Child B cirrhosis, and 28 Child C cirrhosis).

TRF length analysis

Genomic DNA was extracted with phenol-chloroform according to standard protocols. The DNA was rehydrated in TE buffer and an aliquot of undigested DNA was run in an agarose gel to exclude DNA degradation. For TRF length measurement, 4 μg of DNA was digested overnight with *Hinf*I and *RSA*I at 37°C . Complete digestion of the DNA samples was confirmed in a 1.5% agarose gel. Digested DNA was run in a 0.8% TAE gel overnight at 40 volts. Electrophoresis was stopped when the 2 kb size marker had run 14 cm into the gel. The high molecular weight marker and the 1 kb ladder

(Life Technologies, Grand Island, NY) were used as size standards. After 20 min of denaturation and neutralization, the gel was dried for 1 h at 60°C in a vacuum dryer. One hour prehybridization and 4 h hybridization were performed at 37°C . The $(\text{TTAGGG})_3$ oligo-nucleotide was radioactive labeled and used for hybridization. After three washes in 0.25% SSC, 0.1% SDS, the gel was put on a PhosphorImager-screen overnight. The mean TRF length was calculated by measuring the signal intensity in 15 squares covering the entire TRF smear. All calculations were performed with PCbas and Excel (Microsoft) computer programs.

In situ Q-FISH on interphase nuclei

In situ Q-FISH on interphase nuclei was performed as described previously (16, 17). In brief, 7 μm sections were fixed in 4% paraformaldehyde in cacodylate buffer. After three washes in PBS, a second fixation was carried out in 4% formaldehyde, followed by enzymatic unmasking of the sections for 10 min at 37°C (enzyme mix: 100 mg pepsine/50 mg collagenase/100 mg dispase/84 μL concentrated HCl/100 mL water). Fixation and washing steps were repeated as described, followed by dehydration of the slides. After 3 min of denaturation at 80°C , hybridization was carried out for 2 h at room temperature (hybridization mix: (125 μL final volume): 2.5 μL 1 M Tris-Cl, pH 7.2/21.4 μL MgCl_2 [25 mM MgCl_2 /9 mM citric acid/8.2 mM NaH_2PO_4 , pH 7.4]/175 μL deionized Formamid/12.5 μL 10% (w/w) blocking reagent/5 μL 25 $\mu\text{g}/\text{mL}$ PNA Cy3-telomere probe). The slides were washed twice in washing solution I (100 mL final volume: 70 mL formamide/1 mL 1M Tris-Cl, pH 7.2/1 mL 10% BSA stock solution/28 mL water), followed by three washes in washing solution II (15 mL 1M Tris-Cl, pH 7.2/15 mL 1.5 M NaCl/120 μL Tween 20 (0.08% final)/120 mL H_2O). After dehydration, the sections were mounted with 1:1 (v/v) mixed mounting solution with/without DAPI. Pictures were taken at 2500 ms for the Cy3 images and at 100 ms for the DAPI images. To facilitate the identification of different cell types in the cirrhosis samples, hematoxylin/eosin counterstaining was performed on consecutive sections used for Q-FISH analysis. The quantification of the telomere fluorescence intensity was performed using TFL-TELO V1.0, a telomere analysis program developed by P. Lansdorp. To facilitate day-to day comparison, one standard sample was photographed and analyzed for each individual session. To compare the cell type-specific fluorescence intensity of telomere signals between different cirrhosis samples, the mean fluorescence of hepatocytes was set to 100 units and the fluorescence intensities of the other cell types were adjusted using the same calculation factor. In total, the fluorescence intensities of telomere spots were analyzed from 247 hepatocytes, 170 stellate cells, and 26 lymphocytes in 6 cirrhotic samples with severe fibrosis, from 52 hepatocytes in 2 cirrhosis samples with mild fibrosis, and from 78 hepatocytes in 1 noncirrhotic sample.

Senescence-associated β -Gal staining

Senescence-associated β -Gal staining was performed as described previously (18). In brief, 7 μm cryostat sections were fixed in 3% formaldehyde for 3–5 min, followed by three washes in PBS at room temperature. The slides were immersed in freshly prepared senescence-associated β -gal staining solution (1 mg/mL of 5-bromo-4-chloro-3-indolyl β -D galactoside (X-gal) in DMF/40 mM citric acid/sodium phosphate (pH 6.0)/5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM NaCl/2 mM MgCl_2) and incubated at 37°C for 14–16 h. The stained sections were washed

twice with PBS and counterstained for 1 min with eosin. The excess counterstain was removed by two washes in PBS. The samples were analyzed by two independent investigators in a blinded fashion. All samples were stained in triplicate.

RESULTS

Shortening of TRFs is a disease-independent marker of cirrhosis

To test whether telomere shortening is a general marker of cirrhosis independent of its etiology, telomere length was analyzed on a large selection of cirrhosis samples induced by a broad variety of different liver diseases. A total of 96 liver samples derived from explanted livers of patients undergoing liver transplantation at the Medical School Hannover, Germany, during 1993–2001 were used for this study. Noncirrhotic control samples ($n=20$) were derived from patients with acute liver failure, cystic liver disease, or liver surgery due to metastatic liver tumors. Cirrhotic liver samples ($n=76$) were derived from patients with chronic viral hepatitis ($n=27$), autoimmune hepatitis ($n=11$), primary sclerosing cholangitis ($n=20$), primary biliary cirrhosis ($n=13$), and alcoholic liver disease ($n=5$).

The length of TRFs was analyzed using DNA extracted from whole organ samples. The mean TRF length was significantly shorter in cirrhotic livers than with noncirrhotic samples (Fig. 1A; mean TRF length of cirrhotic samples: 7.35 kb, range: 5.7–9.5kb; mean TRF length of noncirrhotic samples: 9.15 kb, range: 7.5–11.5 kb; $P=0.0001$). Telomeres were uniformly short at the cirrhosis stage independent of its etiology (Fig. 1B, C) and each cirrhosis subgroup had significantly shorter TRFs compared with the controls (Fig. 1B), demonstrating that telomere shortening is a disease-independent marker of cirrhosis.

Shortening of TRFs is an age-independent marker of cirrhosis

To further characterize telomere shortening as a general marker of cirrhosis, TRF length of cirrhotic and noncirrhotic samples was correlated to the age of the patients at the time of surgery. The mean age of patients in the control group was 37.6 years (range: 16–62 years). The mean age of patients in the cirrhosis group was 43.1 years (range: 21–66 years). In the different subgroups of cirrhosis, the mean age was as follows: 47.7 years for patients with chronic viral hepatitis (range: 20–65), 29.1 years for patients with AIH (range: 20–41), 51.8 years for patients with alcoholic liver disease (range: 41–59), 36.8 years for patients with PSC (range: 21–60), and 42.4 years for patients with PBC (range: 25–66). In noncirrhotic samples, the mean TRF length showed a significantly age-dependent decline (Fig. 2A, $P=0.0076$). In contrast, TRFs of cirrhotic samples were similarly short at every given age,

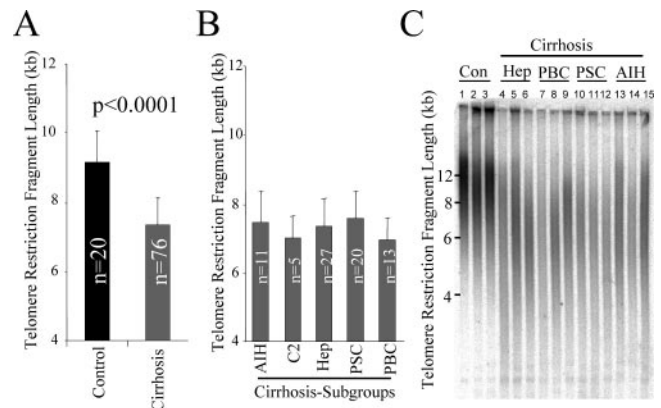


Figure 1. The length of telomere restriction fragments (TRFs) was determined in 76 cirrhosis samples and 20 noncirrhotic controls. *A*) The mean TRF length is significantly shorter in cirrhosis (mean: 7.34 kb) compared with noncirrhotic livers (9.15 kb, $P<0.0001$). *B*) TRFs are uniformly short in cirrhosis subgroups independent of the etiology of cirrhosis: 7.48 kb in autoimmune hepatitis (AIH), 7 kb in alcoholic liver disease (C2), 7.33 kb in chronic viral hepatitis (Hep), 7.57 kb in primary sclerosing cholangitis (PSC, $P=0.001$), and 6.93 kb in primary biliary cirrhosis (PBC). The mean TRF length is not significantly varied in between groups of cirrhosis samples induced by different diseases and each group had significantly shorter TRFs than the noncirrhotic controls. *C*) Representative example of a TRF-Southern blot: noncirrhotic controls (Con) (lanes 1–3), cirrhosis induced by viral hepatitis (Hep) (lanes 4–6), cirrhosis associated with PBC (lanes 7–9), cirrhosis associated with PSC (lanes 10–12), and cirrhosis associated with AIH (lanes 13–15). Note that besides the decrease in mean TRF length, a decrease in the size of the shortest TRFs is present.

showing a weak but not significant age-dependent decrease (Fig. 2D, $P=0.09$). Similarly, none of the cirrhosis subgroups showed a significant age-dependent decrease in TRF length (Fig. 2B–E, data not shown for alcoholic liver disease). Together, these data demonstrate that telomere shortening is an age- and disease-independent marker of cirrhosis.

Masking of telomere shortening during cirrhosis progression by organ architectural changes

To test the correlation between telomere shortening and cirrhosis progression, the grade of cirrhosis was characterized in 72 cirrhosis samples using the Child-Pugh criteria, a clinical score to measure severity of cirrhosis (A, mild cirrhosis; B, moderate cirrhosis; C, severe cirrhosis). Surprisingly, there was no significant correlation between the TRF length and the cirrhosis stage in our survey (Fig. 3A). These data indicate that either telomeres had reached a critically short length at the onset of cirrhosis, not allowing further telomere attrition, or changes in the cellular composition of the cirrhotic liver would affect the average TRF length of whole organ samples. Cirrhosis is characterized by increasing fibrosis of the liver often associated with significant inflammatory infiltration of the organ. These changes in the cellular composition during

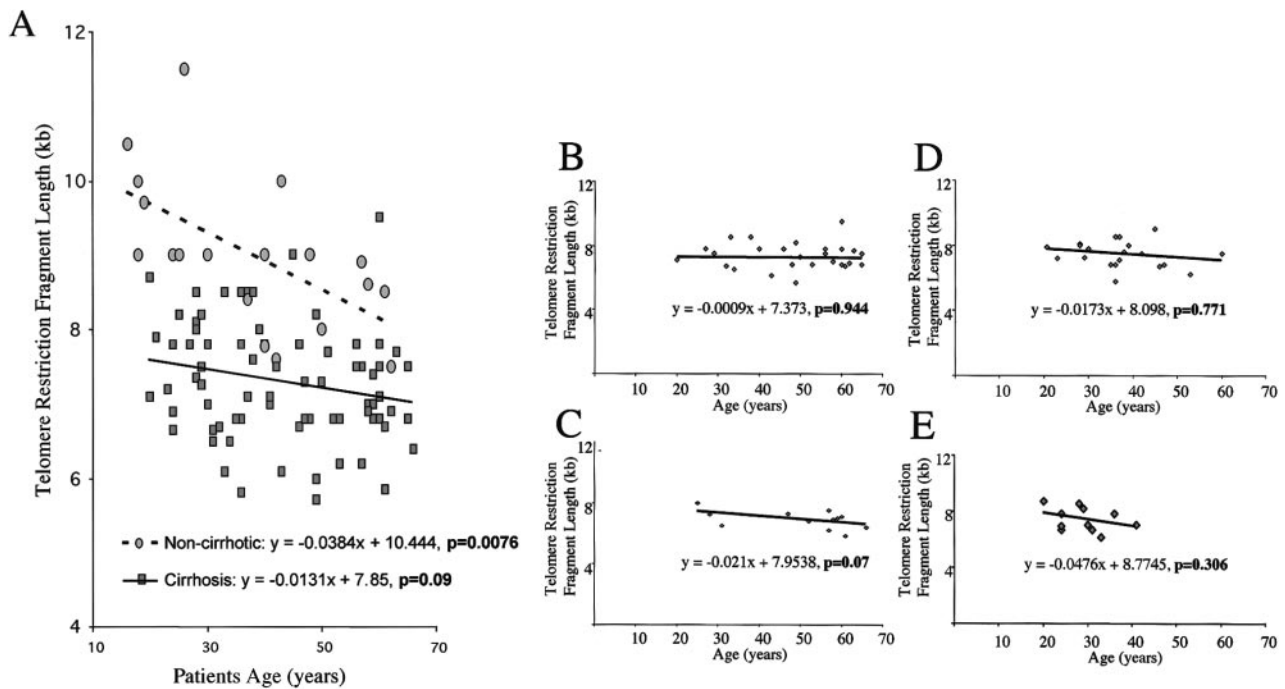


Figure 2. Shortening of TRFs is an age-independent marker of cirrhosis. *A*) The mean TRF length (*y* axis) of individual samples was correlated to the age of the patients (*x* axis). A significant age-dependent decline in mean TRF length of liver samples is present in patients with noncirrhotic livers (ovals, $P=0.0076$). In contrast, TRFs are shorter in cirrhotic samples at every given age, showing only a weak but not significant age-dependent decline (squares, $P=0.09$). *B–E*) None of the disease subgroups of cirrhosis showed a significant age-dependent decline in TRF length: *B*) chronic viral hepatitis, *C*) primary sclerosing cholangitis, *D*) primary biliary cirrhosis, *E*) autoimmune hepatitis. Data not shown for cirrhosis induced by alcoholic liver disease.

progression of cirrhosis could affect the overall telomere length of whole organ samples, possibly counteracting telomere shortening in hepatocytes, the cell type predominantly affected by chronic liver diseases. To test this possibility, the rate of inflammatory infiltration was determined in 52 of the cirrhosis samples using the Ishak classification, a pathological score to qualify inflammatory infiltration in cirrhosis (19). TRFs were significantly shorter in cirrhosis samples showing low inflammatory infiltration compared with cirrhosis samples showing high rates of inflammatory infiltration (mean length: 6.95 kb vs. 7.7 kb, $P=0.006$, Fig. 3*B*). This analysis revealed that within the subgroup of liver samples showing low rates of lymphocytic infiltration, TRFs were significantly shorter in severe cirrhosis (Child C: 6.8kb, Fig. 3*C*) vs. mild cirrhosis (Child A: mean length 7.6kb, $P=0.04$, Fig. 3*C*). Together, these data show that telomere shortening correlates to cirrhosis progression but that changes in the cirrhotic liver, such as lymphocyte infiltration, counteract this correlation.

Hepatocyte-specific telomere shortening in cirrhosis

To directly assess which cell type in the cirrhotic liver shows telomere shortening, quantitative fluorescence in situ hybridization (Q-FISH) on interphase nuclei was performed on frozen sections of cirrhotic livers using a telomere-specific PNA probe (16, 17). In cirrhosis samples, three distinct cell populations are distinguish-

able by cell morphological aspects visualized by counterstaining using DAPI and hematoxylin/eosin solution (Fig. 4*A–C*): 1. Hepatocytes are located in regenerative nodules with round nuclei and a large cytoplasm (Fig. 4*A, B*), 2. Stellate cells appear densely packed in fibrotic septa as elongated cells with elongated nuclei (Fig. 4*A, B*), 3. Lymphocytes in inflammatory infiltrates are characterized by densely packed populations of cells with round nuclei and very small cytoplasm, mainly located within fibrotic septa (Fig. 4*C*). When the fluorescence intensity of telomeres was analyzed specifically in hepatocytes, a significantly weaker mean fluorescence intensity was detected in cirrhosis (mean: 100 units) compared with noncirrhotic controls (mean: 212 units, $P<0.0001$, Fig. 4*D*). Using this method, the difference between cirrhosis and noncirrhotic controls was more pronounced than the difference in mean TRF length detected by Southern blotting (Fig. 1*A*), indicating that telomere shortening in cirrhosis predominantly affects hepatocytes. To further characterize cell type-specific telomere length in cirrhosis the fluorescence intensity of telomeres was compared between hepatocytes, stellate cells and lymphocytes within individual sections of cirrhosis of different etiologies. Independent of the etiology, fluorescence intensity was significantly weaker in hepatocytes (mean: 100 units) compared with stellate cells (mean: 147 units, $P<0.0001$, Fig. 4*D, E*) or lymphocytes (mean: 214, $P<0.0001$, Fig. 4*D*) in all cirrhosis samples tested. Hepatocytes had reduced mean and maximal fluorescence intensities

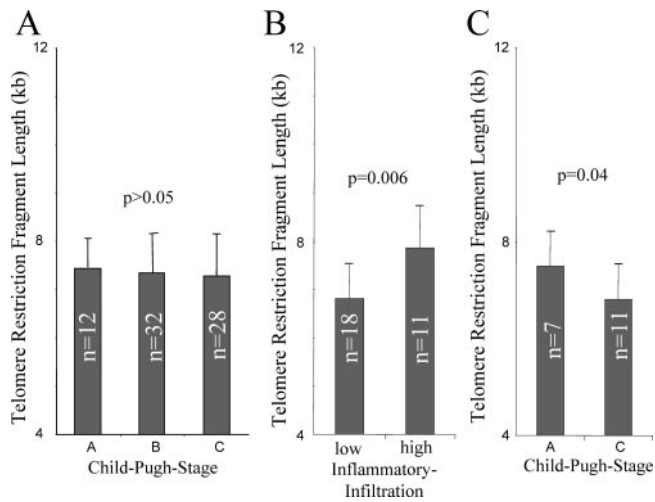


Figure 3. Progressive shortening of TRFs during disease progression is counteracted by organ architectural changes in cirrhosis. *A*) Cirrhosis samples were grouped according to the Child-Pugh criteria: Child A, mild cirrhosis, Child B, moderate cirrhosis, Child C, severe cirrhosis. The mean TRF length is uniformly short in cirrhosis independent of the stage of the disease. *B*) The grade of lymphocytic infiltration was analyzed according to the Ishak-score (19) and the samples were grouped into three categories: mild infiltration, moderate infiltration, and high infiltration. Telomeres are significantly shorter in samples with low inflammatory infiltration (mean TRF length: 6.9 kb vs. 7.7 kb). *C*) The correlation between disease stage (Child-Pugh stage) and TRF length was specifically tested for liver samples with low inflammatory infiltration. In this group TRFs are significantly shorter in advanced cirrhosis (Child C) compared with early cirrhosis (Child A) (mean length: 6.8 kb vs 7.5 kb, $P=0.04$). Together these data indicate that inflammatory infiltration counteracts the shortening of telomeres in whole organ samples of cirrhosis.

compared with the stellate cells and a higher percentage of hepatocellular telomere spots had minimal fluorescence intensities (Fig. 4E). These data demonstrate that telomere shortening in cirrhosis predominantly affects hepatocytes whereas other cell types in the cirrhotic liver have longer telomere reserves.

Hepatocyte-specific senescence in cirrhosis

If telomere shortening limits the regenerative capacity of hepatocytes signs of cellular senescence might be detectable in hepatocytes at the cirrhosis stage. To test this possibility, β -Gal staining was conducted on 49 of the cirrhosis samples and 15 of the control samples. An association between β -Gal activity at pH6 and cellular senescence has first been described for cells in tissue culture (20). An increase in β -Gal-positive keratinocytes in the skin of humans during aging (18) and an increase of β -Gal-positive liver cells in precirrhotic liver affected by chronic viral hepatitis (21) have been described. Nevertheless, background activity of β -Gal not linked to cellular senescence has been reported (22), indicating the need to evaluate β -Gal staining in correlation with telomere length. In our survey, a

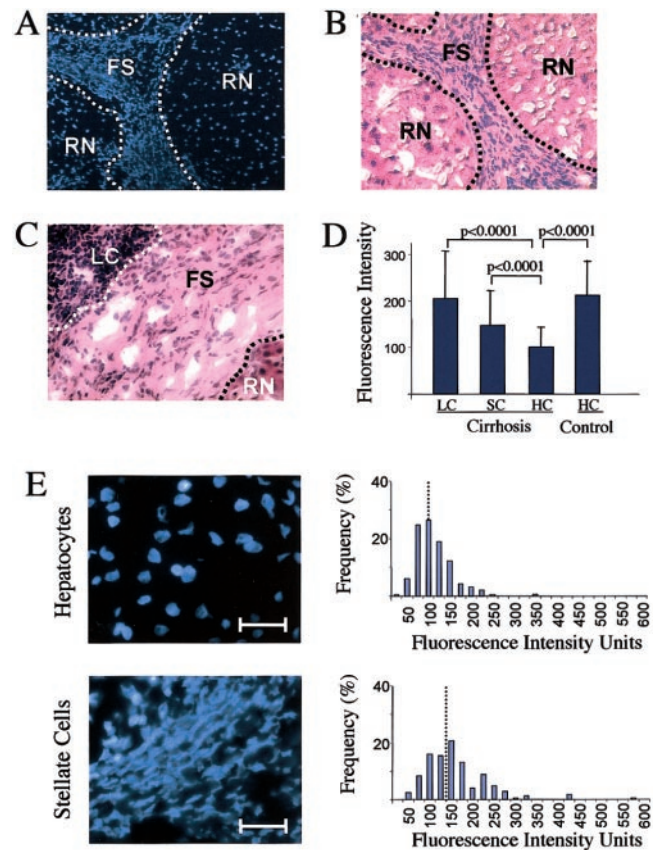


Figure 4. Hepatocyte-specific telomere shortening in cirrhosis. *A*) To identify different cell types in cirrhosis DAPI staining was performed on the same sections that were used for telomere probe hybridization. *B*, *C*) H&E staining was performed on consecutive sections to verify the identification of the different cell type. *A*, *B*) Hepatocytes are located in regenerative nodules (RN) with round nuclei and a large cytoplasm, whereas stellate cells appear densely packed in fibrotic septa (FS) as elongated cells with elongated nuclei. *C*) Lymphocytes (LC) are located in inflammatory infiltrates mostly within fibrotic septa (FS). They are characterized by densely packed populations of cells with round nuclei and very small cytoplasm. *D*) A cell type-specific analysis of the telomere fluorescence intensity was performed on hepatocytes (HC) in control samples and cirrhosis. The telomere fluorescence intensity of stellate cells (SC) and lymphocytes (LC) was analyzed in cirrhosis. The mean fluorescence intensity of hepatocellular telomeres is significantly weaker in cirrhosis compared with noncirrhotic controls. Within cirrhotic samples the fluorescence intensity is significantly stronger in stellate cells (SC) and lymphocytes (LC) compared with hepatocytes (HC). The decrease in telomere length specifically in hepatocytes appears more pronounced compared with the difference in TRF length by Southern blotting, when DNA of whole organ samples is analyzed (Fig. 1A), indicating that longer telomeres in stellate cells and lymphocytes mask the decrease in telomere length in hepatocytes when analyzing whole organ samples. *E*) Pictures of DAPI-stained sections on the left show high magnification of the morphology of hepatocytes located in regenerative nodules (top) and stellate cells located in fibrotic septa (bottom) (magnification bar: 100 μ m). The graphs on the right show the distribution of the fluorescence intensity of telomere spots separately for hepatocytes (top) and stellate cells (bottom) within cirrhotic samples of different etiology. Hepatocytes show reduced mean (dashed line) and maximum values; a higher percentage of cells have minimum fluorescence intensity values (left shift).

strong correlation between senescence-associated β -Gal activity and cirrhosis was detectable: 41 of 49 cirrhosis samples (84%) had β -Gal activity, whereas only 1 of 15 control samples (7%) showed very weak β -Gal activity (Fig. 5A, $P < 0.0001$). β -Gal activity was detectable at a high frequency in all subgroups of cirrhosis: in 90% of the AIH, 75% of the viral hepatitis, 85% of the PSC, 86% of the PBC, and 86% of the cirrhosis samples induced by alcoholism (Fig. 5B). Concordant with our data on hepatocyte-specific telomere shortening in cirrhosis (see above), only hepatocytes stained positive for β -Gal whereas stellate cells in fibrotic septa did not stain positive for β -Gal in any samples tested (Fig. 5C). The β -Gal staining pattern of hepatocytes in cirrhosis is markedly pronounced at the edge of regenerative nodules as opposed to the center of the nodules (Fig. 5C). Since the regenerative nodules in the cirrhotic liver represent clonal expansion of regenerating hepatocytes, the cells at the edge of these nodules have undergone more cell divisions than cells in the center, providing a possible explanation for the increase in senescence-associated β -Gal activity in these regions. Quantification of the percentage of β -Gal-positive hepatocytes within cirrhosis samples showed that 32% of the cirrhosis samples have a weak β -gal activity (<5% of the hepatocytes), 32% have a moderate activity (5–15% of the hepatocytes), and 36% show strong activity (>15% of the hepatocytes). In vitro studies of human fibroblasts have shown that proliferation significantly decreases before end-stage senescence (23) and that the rate of β -Gal-positive cells at this stage is 11% similar to the rate of β -Gal-positive hepatocytes detectable in the vast majority of cirrhosis samples in our survey (68%). Together, our data show that there is a significant rate of hepatocellular senescence in cirrho-

sis limiting the regenerative capacity of the injured organ, thereby perturbing the balance of injury and regeneration, culminating in fibrotic scarring.

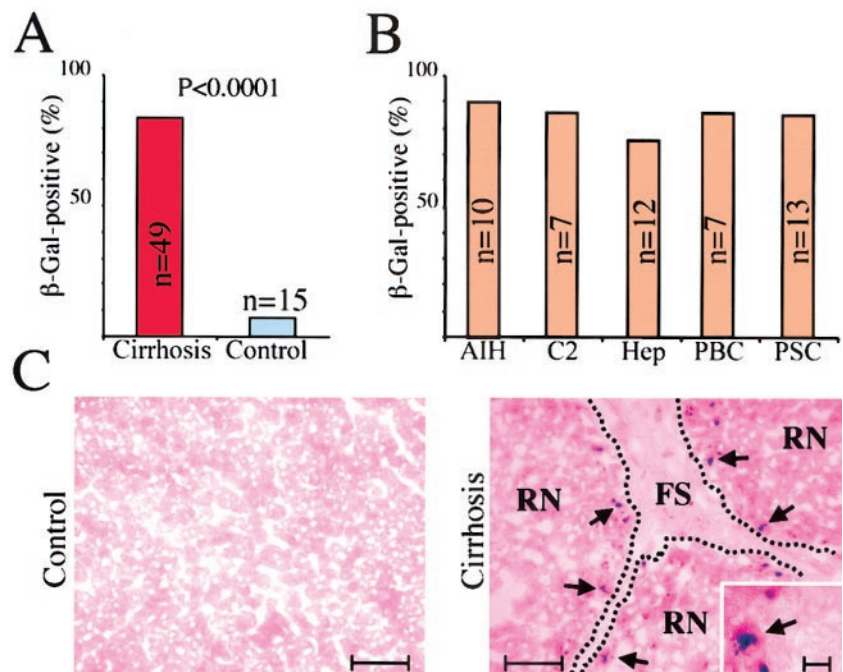
Hepatocyte telomere shortening and senescence correlate to fibrosis progression in cirrhosis

To test the hypothesis that limitation of hepatocyte regeneration by telomere shortening and senescence triggers fibrotic scarring, we evaluated the correlation between hepatocellular telomere length and fibrosis and between the rate of senescent hepatocytes (β -Gal positive) and fibrosis. Cirrhosis samples were grouped into samples with mild fibrosis and samples with severe fibrosis according to the Ishak criteria (19). In line with our hypothesis, this analysis showed that samples with severe fibrosis have significantly shorter telomeres and higher rates of hepatocyte senescence than samples with milder fibrosis (Fig. 6A, B).

DISCUSSION

Our study shows that telomere shortening is a disease- and age-independent sign of liver cirrhosis in humans. Telomere shortening is present in cirrhosis induced by viral hepatitis (chronic hepatitis A and B), toxic liver damage (alcoholism), autoimmunity, and cholestasis (PBC and PSC); telomeres are uniformly short in cirrhosis independent of the age of the patients. We show that telomere shortening and senescence specifically affect hepatocytes in the cirrhotic liver and that both parameters strongly correlate with progression of fibrosis during cirrhosis.

Figure 5. Hepatocyte-specific senescence in cirrhosis. A) 41 of 49 cirrhosis samples (84%) stain positive for senescence-associated β -Gal activity at pH 6 (ref. 20), whereas only 1 of 15 control samples shows very weak β -Gal activity (7%, $P < 0.0001$). B) High rates of senescence-associated β -Gal activity are present in all subgroups of cirrhosis independent of its etiology: autoimmune hepatitis (AIH, 90%), alcoholism (C2, 86%), viral hepatitis (Hep, 75%), primary biliary cirrhosis (PBC, 86%), primary sclerosing cholangitis (PSC, 85%). C) Representative photograph of a noncirrhotic control sample showing no staining for senescence-associated β -Gal activity (left site, Bar: 200 μ m), and a cirrhotic sample showing positive staining for senescence-associated β -Gal activity (right site, bar: 200 μ m). Positive β -Gal staining of cirrhosis samples was restricted to hepatocytes and was not present in other cell types. Inset: High magnification of an area showing hepatocyte-specific β -Gal staining (left site, arrow) at the edge of a fibrotic septum (right site); bar: 30 μ m. β -Gal-positive hepatocytes (arrows) are predominantly located at the edge of regenerative nodules (RN), whereas stellate cells in fibrotic septa (FS) do not show β -Gal activity.



Our data support the telomere hypothesis of human cirrhosis (4, 12), suggesting that chronic hepatocyte damage and concomitant hepatocyte regeneration accelerate telomere shortening in hepatocytes. When hepatocytes reach the senescent stage, liver regeneration decreases but the chronic liver damage continues. At this stage of disease, other cell types, like hepatic stellate cells, which usually do not participate in the regenerative process, become activated and form fibrotic scar tissue in areas of hepatocyte loss (Fig. 7). This model gives a plausible explanation for the long latency of cirrhosis induced by a variety of chronic liver diseases. Further support for this model comes from the observation that hepatocellular proliferation in response to chronic liver injury dramatically decreases at the cirrhosis stage (24–27) and that cell cycle inhibitors like p53 and p21 are overexpressed in cirrhosis (28, 29) similar to the accumulation of p53 in senescent cultures (23, 30). The incidence of accelerated cirrhosis in telomerase-deficient mice (*mTERC*^{-/-}) with short telomeres compared with mice with longer telomeres gives experimental support for telomere hypothesis of human cirrhosis (12).

Our study implicates that an effective treatment of disease stages associated with telomere shortening during aging (7) and chronic high turnover diseases (8–11) would require the targeting of a specific cell type within the affected organ. The data implicate that a hepatocyte-directed therapy to restore telomere length could potentially rescue cirrhosis in chronic liver diseases. In line with this hypothesis, telomerase gene delivery by adenovirus vectors prevents cirrhosis

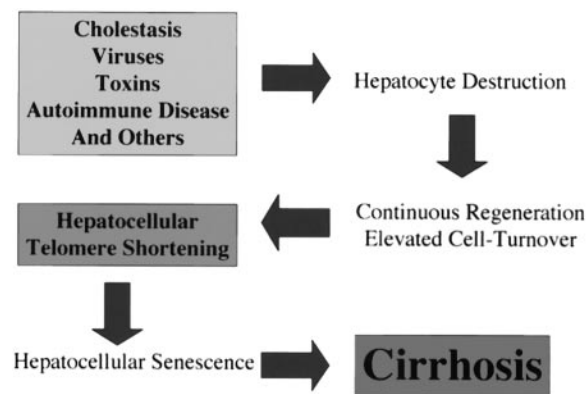


Figure 7. The telomere hypothesis of cirrhosis. In chronic liver disease, continuous liver damage and concomitant regeneration accelerate telomere shortening, specifically in hepatocytes finally culminating in senescence and cessation of the regeneration. Continuous liver damage at this point triggers the activation of stellate cells and fibrotic scarring.

formation in mice with short telomeres (12). Nevertheless, it remains to be explored to what extent cirrhosis can be rescued in advanced stages of the disease. Since cellular senescence is generally considered to be irreversible, an activation of telomerase in end-stage cirrhosis could come too late. A potential downside of telomerase therapy could be an elevated risk of liver cancer, which is associated with telomerase reactivation in > 80% of the cases (31). It has been shown that telomere shortening inhibits tumorigenesis in *mTERC*^{-/-} mice that retain functional p53 (17, 32, 33).

A careful evaluation of a telomerase therapy for cirrhosis is needed. It will be important to identify the signals inducing hepatocellular senescence once telomeres have reached a critically short length. The tumor suppressor p53 has been identified as a downstream target of short dysfunctional telomeres in mouse (34) and human cells (35). Inhibition of p53 rescues the adverse effects of telomere dysfunction (34). The data on accumulation of p21 in cirrhotic samples (28, 29) indicate this pathway might also be activated at the cirrhosis stage. However, the mechanism of p53 activation and the role of other pathways in response to critical telomere shortening remain to be identified. The detailed characterization of such signals will possibly identify new targets besides telomerase for the treatment of cirrhosis, other chronic disease and disease stages associated with loss of regenerative capacity during aging. EJ

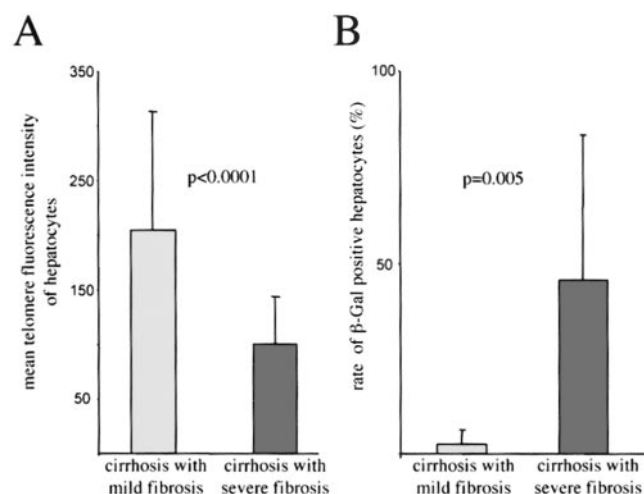


Figure 6. Correlation of telomere length and β -Gal activity with fibrosis progression. Cirrhosis samples were grouped into samples with mild fibrosis and samples with severe fibrosis according to the Ishak criteria (19). *A*) There was a significant difference in mean telomere length in these groups showing that fibrosis progression correlated with significant telomere shortening in hepatocytes. *B*) In line with the data on telomere length there was a significant increase in the percentage of hepatocytes staining positive for β -gal activity in cirrhosis samples with severe fibrosis compared with cirrhosis samples with mild fibrosis.

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