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Human Telomerase Activity, Telomerase and Telomeric Template Expression in Hepatic Stem Cells and in Livers from Fetal and Postnatal Donors

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Abstract

Background—Even though telomerase activity has been analyzed in various normal and malignant tissues, including liver, it is still unknown to what extent telomerase can be associated with specific maturational lineage stages.

Methods—We assessed human telomerase activity, protein and gene expression for the telomerase reverse transcriptase, as well as expression of the telomeric template RNA hter in hepatic stem cells and in various developmental stages of the liver from fetal to adult. Additionally, the effect of growth factors on telomerase activity was analyzed in hepatic stem cells *in vitro*.

Results—Telomerase was found to be highly active in fetal liver cells and was significantly higher than in hepatic stem cells, correlating with gene and protein expression levels. Activity in postnatal livers from all donor ages varied considerably and did not correlate with age or gene expression levels. The hter expression could be detected throughout development. A short stimulation by growth factors of cultured hepatic stem cells did not increase telomerase activity.

Conclusions—Telomerase is considerably active in fetal liver and variably in postnatal livers. Although telomerase protein is present at varying levels in liver cells of all donor ages, gene expression is associated solely with fetal liver cells.

Keywords

telomerase; htert; hter; hepatoblast; hepatic stem cell; hepatocyte

Introduction

The ribonucleoprotein telomerase is a reverse transcriptase adding telomeric repeat sequences to the end of chromosomes (1), the telomeres, thereby protecting chromosomes from shortening. Telomeres have been shown to shorten with each cell cycle, predicting the cell's replicative capacity (2). Most somatic cells do not express telomerase activity and, therefore,

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have a limited life span. In contrast, the majority of cancer cells, tumor cell lines, germ cells, and certain stem cells of the adult do express high telomerase activity resulting in telomere extension, correlating with extremely extended replicative capacity (3). Induced depression of telomerase *in vivo* in highly proliferative organs like testis and bone marrow, as well as in cell cultures leads to an increase in apoptosis, decrease in proliferation, as well as loss and fusion of chromosomes (4).

The adult liver comprises parenchymal cells, the hepatocytes and cholangiocytes, as the major, highly differentiated cell types, and non-parenchymal cell type types that include endothelial cells, stellate cells, Kupffer cells and hematopoietic cells (5–10). Within all fetal and postnatal livers, there are hepatic progenitors that include hepatic stem cells and their immediate descendents, the hepatoblasts (11,12), hypothesized to be transit amplifying cells (13). Both hepatic stem cells and hepatoblasts express epithelial cell adhesion molecule (EpCAM). During development from fetal through adult stages, the percentage of hepatic stem cells (0.5–1.5%) remains constant. In fetal liver the parenchymal population is comprised of hepatoblasts. In adult livers the major parenchymal populations are the hepatocytes and cholangiocytes. Thus, sorting for EpCAM+ cells from fetal liver cell suspensions results in an enriched hepatoblast population, whereas when done from suspensions of adult liver cells results in an enriched hepatic stem cell population. During fetal development, the maximum growth of liver is observed, with an 84-fold increase in volume from gestational day 13 to 20 in rat (14). We have shown previously (11,12,15) that selection for human hepatic stem cells (hHpSCs) from fetal and adult liver *in vitro* is possible by either immunoselection or by plating liver cells on plastic and maintaining cultures in a serum-free, hormonally defined medium tailored for hepatic progenitors, Kubota's Medium (16). Under these conditions, stem cell colonies are formed that can be collected selectively.

Under non-pathological conditions, the adult liver represents a quiescent organ, with very low cell turnover. A unique property of the liver is its ability to regenerate in either of two distinct ways: regeneration after partial hepatectomy (PH) versus that after selective loss of mature parenchymal cells in zone 3 (and sometimes also in zone 2) of the liver acinus (17,18). After PH, the majority of the liver cells undergo DNA synthesis beginning in the portal triad region and proceeding to the pericentral area by 36 to 48 hours (19). Epidermal growth factor (EGF) and hepatocyte growth factor (HGF) signaling are up-regulated after PH within one hour (EGF) or even minutes (HGF) (20). The complete liver mass is restored within a week. Increased telomerase activity has been observed 24h after PH in the total parenchymal cell population of mice (21) and pigs (22); pretreatment of mice with EGF and HGF resulted in increased telomerase activity *in vivo* after PH.

In regeneration following loss of cells in zone 3 (and sometimes also zone 2) due to viruses, radiation or drugs, there is proliferation of periportal cells (zone 1) followed by rapid differentiation to cells with phenotypes of cells in zone 2 and 3; the phenomenon has been described in livers of most mammalian species and is referred to as the "oval cell response" alluding to the presence of large numbers of cells, shown to be progenitors and having oval shaped nuclei (23). In both forms of liver regeneration, it remains unclear to which extent one or more of the known maturational lineage stages of the hepatic parenchymal cells contribute to the regenerative process, and show telomerase activity or its activation in response to growth factor stimulation.

Importantly, many studies analyzing telomerase expression by immunohistology in normal and pathological conditions have to be re-evaluated as it has been demonstrated that various commercially available antibodies do not react specifically to telomerase (24).

In this study we analyzed telomerase activity, protein and gene expression for hTERT, as well as expression of the telomerase RNA component hTER in cultures of human hepatic stem cells, and compared these to the enriched parenchymal cell preparations from fetal and postnatal livers. Also, we examined whether induced proliferation in hepatic stem cells by short-term stimulation with EGF and HGF has an effect on telomerase activity in stem cells *in vitro*.

Materials and Methods

Adult Liver Cell Isolation

Suspensions of human adult liver cells were obtained from Vesta Therapeutics (Research Triangle Park, NC): Donated livers, not suitable for orthotopic liver transplantation, were acquired from federally designated organ procurement organizations. Informed consent was obtained from next of kin for use of the livers for research purposes. No organs were obtained from executed prisoners or other institutionalized persons. To isolate cells, livers were perfused through the portal vein and hepatic artery with EGTA-containing buffer for 15 min and 125 mg/l Liberase (Roche, Indianapolis, IN) for 30 min at 34°C. The enzymatically digested tissue was mechanically dissociated by raking the tissue by hand, and the resulting cell suspension was passed sequentially through filters of pore size 1,000, 500, 250, and 150 μm, and centrifuged in Optiprep (Axis-Shield, Oslo, Norway) density gradients at 500g to select for viable cells.

Fetal Liver Cell Isolation

Fetal livers obtained from Advanced Biological Resources (Alameda, CA) were of gestational stages between 16 and 20 weeks. Cells were isolated as described previously, including slow-speed centrifugations to reduce numbers of hematopoietic cells (12,15).

Cell Cultures

The hHpSCs *in vitro* were obtained by culture selection methods as described previously (12,15). Briefly, fetal liver cells obtained by collagenase digestion were seeded in plastic culture dishes (Falcon/Becton Dickinson, Franklin Lakes, NJ) and cultured in serum-free Kubota's medium (supplemented RPMI 1640 (Gibco/Invitrogen, Carlsbad, CA)) selective for hHpSCs (16) with an initial 24 h phase of 10% fetal bovine serum (Gibco/Invitrogen). Thereafter, serum free medium was changed every fourth day. For telomerase activity measurement and expression studies, hHpSC colonies were picked selectively after 2 – 3 weeks in culture, washed once with PBS, and pellets were flash frozen on dry ice and kept at −80°C for later analyses. For immuno-cytochemical analyses, hHpSC colonies were fixed on culture dishes.

HEPG2 and HeLa cells (Tissue Culture Facility at University of North Carolina Lineberger Comprehensive Cancer Center, Chapel Hill, NC) were grown in DMEM supplemented with 10% fetal bovine serum.

Cell Sorting

Enriched populations of hHpSCs or of hepatoblasts (the *in vivo* populations) were obtained as EpCAM+ cells immunoselected from freshly prepared adult and fetal liver cell suspensions using magnetic activated cell sorting according to manufacturer's instructions (Miltenyi Biotec, Auburn, CA) as described previously (12). Cells were incubated in the dark at 4°C with FITC-conjugated anti-EpCAM antibody (Miltenyi Biotec) for 20 min at a concentration of 50 μl for 50×10^6 total cells in 500 μl buffer (phosphate buffered saline (PBS) containing 0.5% bovine serum albumin and 2 mM EDTA (Sigma)). EpCAM+ cells were labeled with magnetic beads using an anti-FITC Multisort Kit (Miltenyi Biotec) and selected by Midi- or MiniMACS

columns and separation units (Miltenyi Biotec). All steps were performed at 4°C with addition of 10% accutase (Innovative Cell Technologies, San Diego, CA), except antibody incubations, to prevent aggregation of cells.

Supplementation with Growth Factors

Colonies of hHpSCs were incubated for 24 h with 5 ng/ml HGF and 10 ng/ml EGF (PrePro Tech, Rocky Hill, NJ) in Kubota's medium, controls were cultured without addition of growth factors. For telomerase activity measurement, stem cell colonies were picked as described. For proliferation analysis, cultures were treated as described as follows.

Proliferation Analyses

Proliferation rate of hHpSC colonies in culture with and without growth factors was analyzed using the BrdU Labeling and Detection Kit I (Roche, Indianapolis, IN) with 4 h BrdU incorporation time at 37°C; nuclei of all cells were stained with DAPI (Sigma, St. Louis, MO). Stained colonies were photographed using a Leica DMIRB inverted microscope (Leica Microsystems, Bannockburn, IL) with OrcaER camera (Hamamatsu, Hamamatsu, Japan) controlled by SimplePCI (Compix Imaging Systems, Sewickley, PA) software. Percent positive labeled cells (BrdU positive cells per total DAPI cells) within an hHpSC colony were calculated using the MetaMorph software version 6.3r6 Cell Scoring function (Molecular Devices, Downingtown, PA) and compared to controls without growth factor addition.

Telomerase Activity Measurement

Telomerase activity was measured using the TeloTAGGG Telomerase PCR ELISA kit (Roche Diagnostics, Mannheim, Germany). Extracts from human embryonic kidney (HEK)293 cell line included in the kit and HepG2 served as positive controls; Negative controls included heat-treated HEK293 cell extract, DNase-free RNase (Invitrogen) treated cell extracts, and lysis buffer prepared as recommended in the kit. For ELISA readings absorbance was measured at 450 nm against a reference of 630 nm using a SynergyHT plate reader (Biotek, Winooski, VT). Total protein concentrations of extracts were analyzed by Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Data were calculated as absorbance 450nm–630nm per μg total protein, and given as percent of positive control HEK293.

Reverse-Transcriptase Polymerase Chain Reaction

Expression of the human telomerase gene hTERT and the human RNA component of telomerase enzyme hTER were analyzed by RT-PCR. Expression levels were normalized to housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase. RNA was extracted from cells using RNeasy Mini Kit (Qiagen, Valencia, CA) with on-column DNA-digestion, and 500 ng total RNA was reverse transcribed using Superscript II reverse transcriptase and random primers (Gibco/Invitrogen). PCR was performed using Platinum Taq DNA Polymerase (Gibco/Invitrogen) (see Table 1). Products were electrophorized on 2% agarose gels, stained with ethidium bromide (Gibco/Invitrogen) and analyzed using the UVP BioDoc-It System (UVP, Upland, CA).

Western Blots

Protein extracts from cells used for telomerase activity measurements were used to analyze protein expression of htert. Denaturing 7% SDS-PAGE and Blots were done according standard protocols. Nitrocellulose membranes (Bio-Rad) were incubated with primary htert rabbit antibody (Rockland, Gilbertsville, PA) and β-actin mouse IgG1 antibody (Novus Biologicals, Littleton, CO) as loading control. AlexaFluor680 goat anti-rabbit (Molecular Probes/Invitrogen, Carlsbad, CA) and IRDye800 goat anti-mouse (LI-COR, Lincoln, NE) were used as secondary antibodies. Membranes were scanned with an Odyssey infrared scanner

using software version 1.2 (LI-COR). HEK293 were used as positive controls and applied at three concentrations within linear range. Band densities were calculated, and data are given as htert expression normalized to β-actin.

Immuncytochemistry

Colonies of hHpSCs after 12 to 19 days in culture (originating from three different livers) and HepG2 (positive controls) were washed with PBS, fixed with 4% para-formaldehyde in PBS for 30 min, washed and permeabilized with 0.5% NP-40 (ICN Biomedicals, Aurora, OH) in PBS for 10 min. Cells were washed, and endogeneous peroxidases were quenched with 1% H_2O_2 in PBS for 90 min. Cells were washed, and blocked for 1 h at RT with blocking buffer (0.5% bovine serum albumin 0.2% cold water fish gelatin (Sigma)). Rabbit primary antibody against htert (Rockland) was applied in blocking buffer diluted 1:500 for 1 h at RT. In parallel, negative controls without addition of primary antibody for detection of non-specific binding of secondary antibody, and isotype controls with a concentration corresponding to primary antibody were performed using rabbit IgG (R&D Systems, Minneapolis, MD) for detection of non-specific binding of primary antibody. Cells were washed three times with PBS, and secondary labeling was done using the Tyramide Signal Amplification Kit #14 (Molecular Probes/Invitrogen) containing horseradish-peroxidase goat anti-rabbit IgG and Alexa Fluor 568 tyramide for signal amplification. Nuclei of all cells were stained with DAPI (Sigma). Cells were mounted in glycerol-gelatin (Sigma) and photographed using a Leica DMIRB inverted microscope (Leica Microsystems, Bannockburn, IL) with OrcaER camera (Hamamatsu, Hamamatsu, Japan) controlled by SimplePCI (Compix Imaging Systems, Sewickley, PA) software.

Statistical Analyzes

Analyzes of significance were done using the two-tailed Student's t-test. Telomerase activity measurement samples comprised the following: data were analyzed from fetal liver-derived hepatic stem cell colonies from eight different donors; total fetal liver cells were from four different donors; fetal liver EpCAM+ cells from four different donors, and adult liver EpCAM + cells from four different donors. Each extract from every one of the eight or four donors, respectively, was measured twice in the assay giving an average single data point from each donor. Single data points from the eight (n=8) or four (n=4) donors, respectively, were used to calculate a mean activity for fetal liver-derived hepatic stem cell colonies or fetal liver cells. Total liver cell samples from postnatal and adult donors were available from donors of widely varying ages (from 10 month to 68 years). However, no samples were available from more than one donor of a certain age. Therefore, data are given from one single donor of a certain age each, with an average from three different measurements.

Results

Telomerase Activity

Telomerase activity was measured in hHpSCs (colonies and EpCAM+ sorted adult liver cells) and compared to that in enriched hepatoblasts (EpCAM+ sorted from fetal liver cell suspensions) and from parenchymal cell preparations from fetal and postnatal donors of different ages (Fig. 1).

The hHpSC colonies in culture showed detectable but very low enzyme activity (4.0 ± 0.2% of HEK293 [Mean ± SEM]). Still low, but higher activity could be observed in EpCAM+ sorted from adult liver cell suspensions (8.5 ± 2.3% of HEK293), the enriched hHpSC population found *in vivo*. In contrast, the enriched hepatoblast population (EpCAM+ sorted from fetal liver cell suspensions) and parenchymal cell preparations from fetal livers (in which the dominant cell populations are hepatoblasts) demonstrated significant higher telomerase

activities: These were about 15-fold (59.7 ± 15.1% of HEK293) and 5-fold (19.6 ± 1.3% of HEK293) higher than those in hHpSCs colonies in culture ($p<0.0005$ and $p<0.00005$, Student's t-test), respectively, and thus about 7-fold and 2-fold higher than those in the enriched hHpSCs sorted from freshly isolated liver cell suspensions (EpCAM+ adult liver cells) ($p<0.05$ and $p<0.05$, Student's t-test).

Measurements from hHpSC and fetal liver cells were done in duplicates, with at least 20 hHpSC colonies from eight different donors each (n=8), and with fetal liver cells from four different donors (n=4); hHpSC and fetal liver cells demonstrated very low variation in enzyme activity between different donors.

Next, telomerase activity was analyzed in enriched parenchymal cell preparations from postnatal livers of neonatal, pediatric, and adult donors. Enzyme activity was highly variable between donors and did not seem to correlate with donor age. The mean telomerase activity from these eight donors was 18.3 ± 8.0% of HEK293. Measurements were done in triplicate and were averaged to provide data on a single donor (n=1) of a specific age in Figure 1.

Negative controls included lysis buffer, heat-treated extract, and RNase-treated extract, and showed negligible background by reducing telomerase activity to less than 1.6 (heat treatment) and 8% (RNase treatment) of original values.

Gene Expression Analyses of hTER and hTERT

As telomerase enzyme activities in enriched parenchymal cell preparations from postnatal livers were quite variable between donors, we asked whether gene expression for either the telomerase enzyme (hTERT) or the telomeric template RNA (hTER) would correlate with developmental stage. Interestingly, the hTERT gene was expressed only in fetal liver cells (Fig. 2). No expression was observed in enriched parenchymal cell preparations from postnatal livers of all donor ages and nor in hHpSC colonies. By contrast, the telomeric template, hTER, was expressed in hHpSCs and in enriched parenchymal cell preparations from postnatal livers of all donor ages.

Protein Expression Analyses of hTERT

Telomerase activity was observed even though no hTERT expression was detected. Therefore, we analyzed protein expression of htert (Fig. 3) from cell extracts used for activity measurements. In contrast to hTERT gene expression, protein expression correlated much more to enzyme activity, although not always proportionally. In addition, htert protein concentrations of liver cells relative to HEK293 cells were much higher than htert enzyme activities of liver cells relative to HEK293 cells in all analyzed samples.

Immuno-cytochemistry for Telomerase

To confirm protein expression of telomerase enzyme, we performed immuno-cytochemistry for telomerase on hHpSC colonies in culture (Fig. 4). The cells within an hHpSC colony showed unequal expression of telomerase, with some cells near the perimeter of the colony showing expression but usually absent or low level of expression in cells in the center of the colony. Telomerase expression was observed in the nuclei; however, occasionally, very few hHpSCs within a colony demonstrated cytosolic telomerase expression. Isotype and negative controls did not stain.

Effects of Growth Factors on Telomerase Activity and Proliferation in hHpSCs

EGF and HGF are growth factors known to play an important role in liver development and after PH. To assess whether these growth factors have an effect on telomerase activity on cultures of hHpSCs, colonies were incubated with EGF and HGF at concentrations similar to

those appearing after PH (21). Controls of hHpSCs without growth factors indicated that 7.3% of the hHpSCs were positively labeled after 4h BrdU incubation. The addition of EGF and HGF resulted in a slight but significant 1.7-fold increase in proliferation to 12.5% (Student's t-test $p<0.01$). However, this slight induced proliferation was not associated with a significant increase in telomerase activity in hHpSCs (4.0% of HEK293 controls vs. 3.4% of HEK293 induced; Student's t-test $p>0.63$).

Discussion

High telomerase activity has been associated with a variety of cancers, germ cells and stem cells, whereas most differentiated somatic cell types usually have low or non-detectable levels of telomerase activity. In liver, hepatocellular carcinomas have been shown to express high telomerase activity (25–28). However, considerable telomerase activity has been detected in normal adult human liver tissue adjacent to tumors, having about 4–8-fold higher activities (29). In adult normal mice, telomerase activities were often higher than in tumors (30). Telomerase activity in livers from rats from fetal to adult (31) has been demonstrated to be highest in fetal liver and to decrease after birth, and to increase gradually thereafter. Of various organs assayed in 2-years-old rats, only liver showed high telomerase activity.

In general, telomerase activity has been associated with cell proliferation rather than malignant transformation (32,33). The results from our study suggest telomerase in non-pathological livers is associated with the highly proliferative hepatoblasts and a subpopulation of the hHpSCs found at the periphery of the hHpSC colonies but not in the quiescent parenchymal cells in postnatal livers as clearly demonstrated at the gene expression level. This is consistent with findings that stem cells cycle very slowly in contrast to transit amplifying cells of fetal liver (11). Telomerase enzyme activity did not correlate necessarily to protein or gene expression levels, such that hTERT gene expression was only observed in fetal livers. This was true, though telomerase protein was found in parenchymal cells of all donor ages. Thus, hTERT gene expression rather than enzyme activity correlates with cell proliferation.

Measures of human telomerase activity, as opposed to activity in non-human species, are likely to be influenced by donor history including medications (e.g. drug administration, alcohol and nicotine consumption) or pathological conditions. This can make interpretation of some of the data difficult.

Interestingly, substantial activity and protein expression was observed in adult liver parenchymal cells, although no hTERT expression could be detected. This implicates that telomerase protein is stored and stabilized throughout life without *de novo* gene expression under normal conditions. It is unknown if telomerase gene expression is induced in human livers in regenerative responses as has been shown in mice after PH (21), or whether alternative mechanisms for telomere elongation might operate in human livers. For example, telomere elongation might takes place through telomere recombination ["alternative lengthening of telomeres" (ALT) (34)]. Whether this mechanism is operative in human livers needs to be evaluated. In addition, it has been reported that alternative splicing (35–37) and intracellular location (38,39) of the enzyme are important factors influencing telomerase activity. In our findings, the telomerase protein was found consistently in the nuclei in fetal parenchymal cells and in the cytoplasm of parenchymal cells from postnatal livers. Therefore, it is possible that induction of telomerase activity in postnatal parenchymal cells could involve translocation of the protein from the cytoplasm to the nucleus.

To address the issue of alternative splicing in our study we intentionally used a primer pair located downstream of known splice variants of hTERT, thus, detecting all variants. Fetal human liver contains all four known variants (40), findings we confirmed in our studies (data

not shown). We could not detect any expression of full length or splice variants in hepatic stem cell colonies and in enriched hepatic parenchymal preparations from postnatal livers from neonatal to adult donors.

The liver represents a unique organ with its known regenerative capacity following PH and in response to toxic injuries such as drugs, radiation, and hepatitis viruses (41,42). Also, H^3-thymidine and BrdU incorporating studies have demonstrated that all cells of the liver are able to undergo DNA synthesis following PH or toxic injuries (17,19). The observed DNA synthesis is followed by cytokinesis leading to complete cell division in all cells after toxic injury, but cytokinesis occurs in only a subpopulation of the parenchymal cells following PH. PH is associated with an increase in overall telomerase activity in mouse and pig (21,22). In our studies we show that hHpSCs do not increase telomerase activity in response to short exposure times to growth factors. The highest levels of telomerase activity in fetal livers are found in hepatoblasts, the presumptive transit amplifying cells of the liver.

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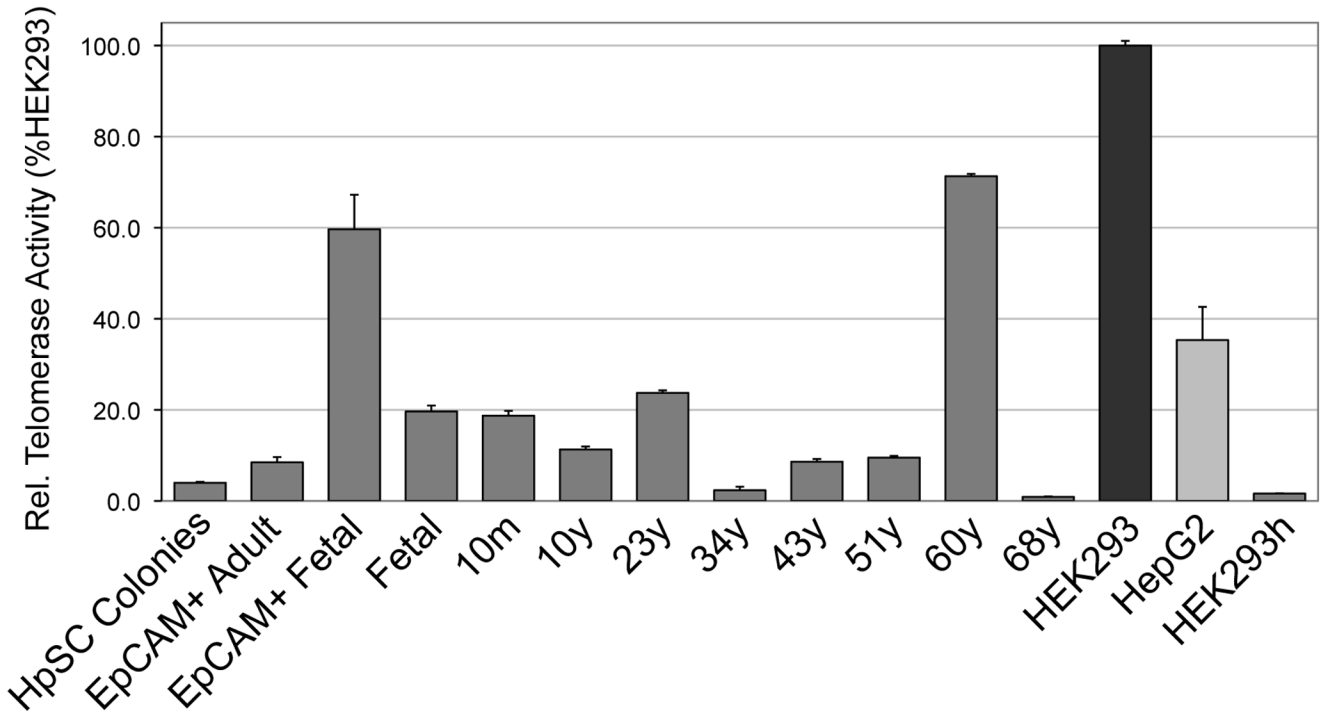

Figure 1. Telomerase Activity during Hepatic Development

Activities were measured in hHpSC: colonies in culture (n=8, duplicate measurements) and sorted EpCAM+ cells from adult liver cell suspensions (n=4, triplicate measurements); in enriched hepatoblast populations: sorted EpCAM+ from fetal liver cell suspensions (n=4, triplicate measurements) and enriched parenchymal cell preparations from fetal livers (n=4, duplicate measurements); and in liver cells from donors of various ages from neonates (10 months) to an elderly adult (68 years) (n=1, triplicate measurements). HEK293 and HepG2 cells served as positive controls; heated HEK293 extract, RNAse treated cell extract, as well as lysis buffer (not shown) served as negative controls. Negative controls showed minimal background of less than 1.6% (heat treatment) and 8% (RNase treatment) of original acitvity (n=3, triplicate measurements). Row data (absorbance 450nm against a reference of 630 nm) were normalized to µg total protein and given as percent of positive control HEK293. Data are given as means ± SEM with variations between different donors for hHpSC colonies and fetal liver preparations, and with variations between different measurements from a single postnatal donor. m: months; y: years; HEK293: human embryonic kidney cell line 293; h: heated; HpSC: hepatic stem cell; EpCAM: epithelial cell adhesion molecule.

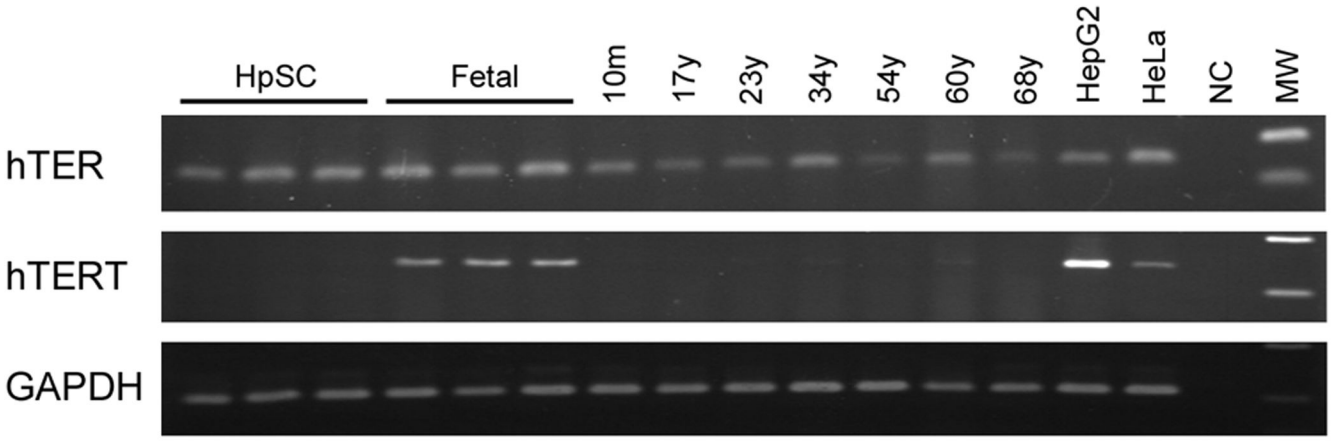

Figure 2. Gene Expression for hTER and hTERT

Colonies of hHpSC, enriched parenchymal cell populations from fetal livers (from three different donors each), and enriched parenchymal cell populations from postnatal donors of various age were analyzed for the expression of the template RNA hTER and for gene expression of the catalytic protein subunit hTERT by RT-PCR. Expression of template RNA hTER could be detected in hHpSCs and in enriched parenchymal cell populations from fetal to adult livers. Only enriched parenchymal cell populations from fetal liver cells expressed RNA for hTERT. HepG2 and HeLa cells were used as strong and weak positive controls, respectively; no template (water) served as the negative PCR control (NC). GAPDH was used as common gene for equal expression control. MW: molecular weight marker; m: months; y: years.

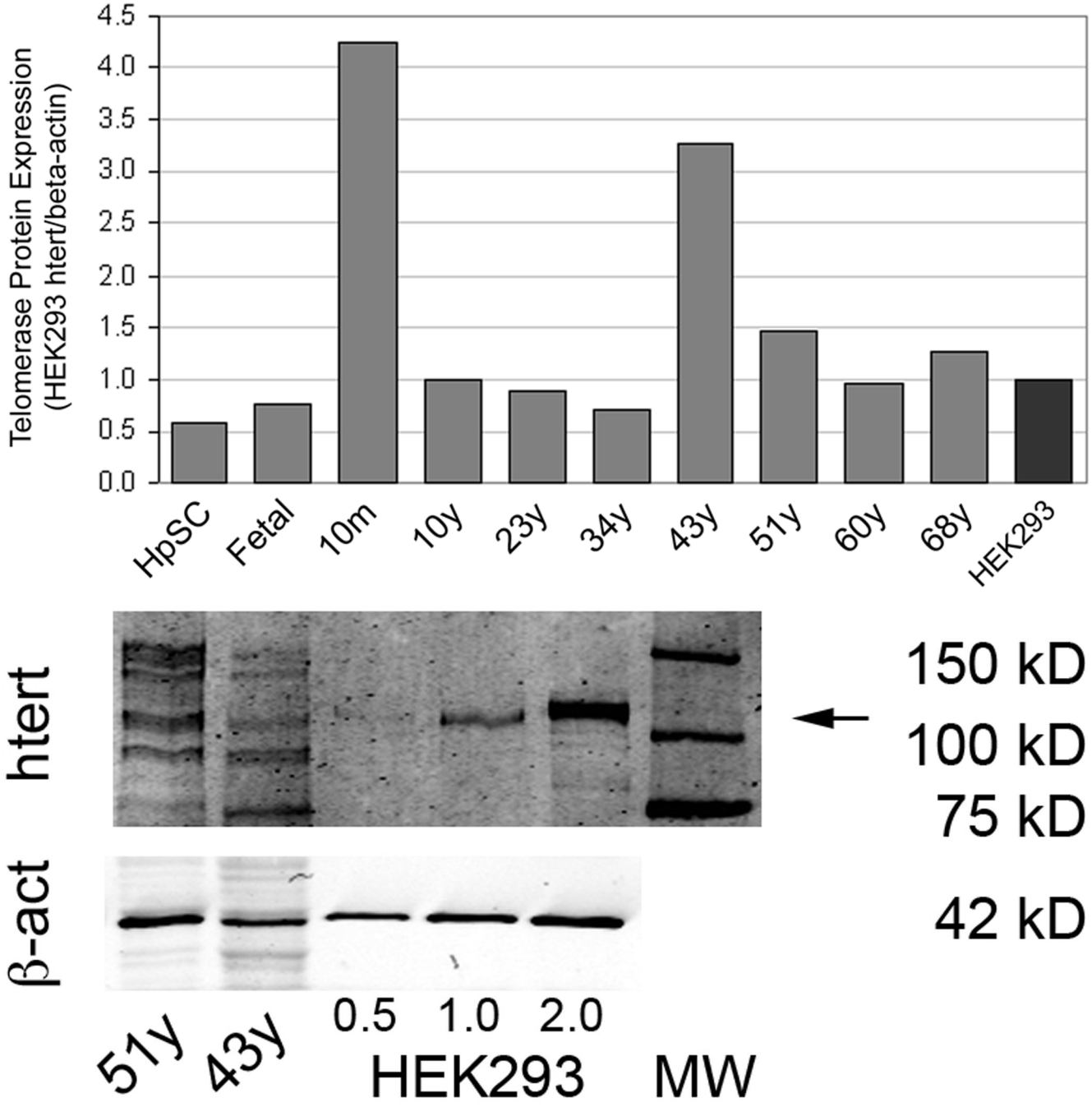

Figure 3. Protein Expression of Telomerase

A: Extracts of hHpSC colonies and enriched parenchymal cell populations from fetal livers (from three different donors each) versus postnatal livers of donors of various age were analyzed for expression of htert protein by Western-Blot. β-actin was used as a loading control, and data are given as the ratio of htert to β-actin, normalized to HEK293 expression. **B:** Example of original band images obtained from a primary scan of the membrane, with HEK293 extract applied at three different concentrations (given as μg of total protein); Arrow is pointing at the expected size of htert (127kD); Upper lane: htert; Bottom lane: β-actin. MW: molecular weight marker; m: months; y: years.

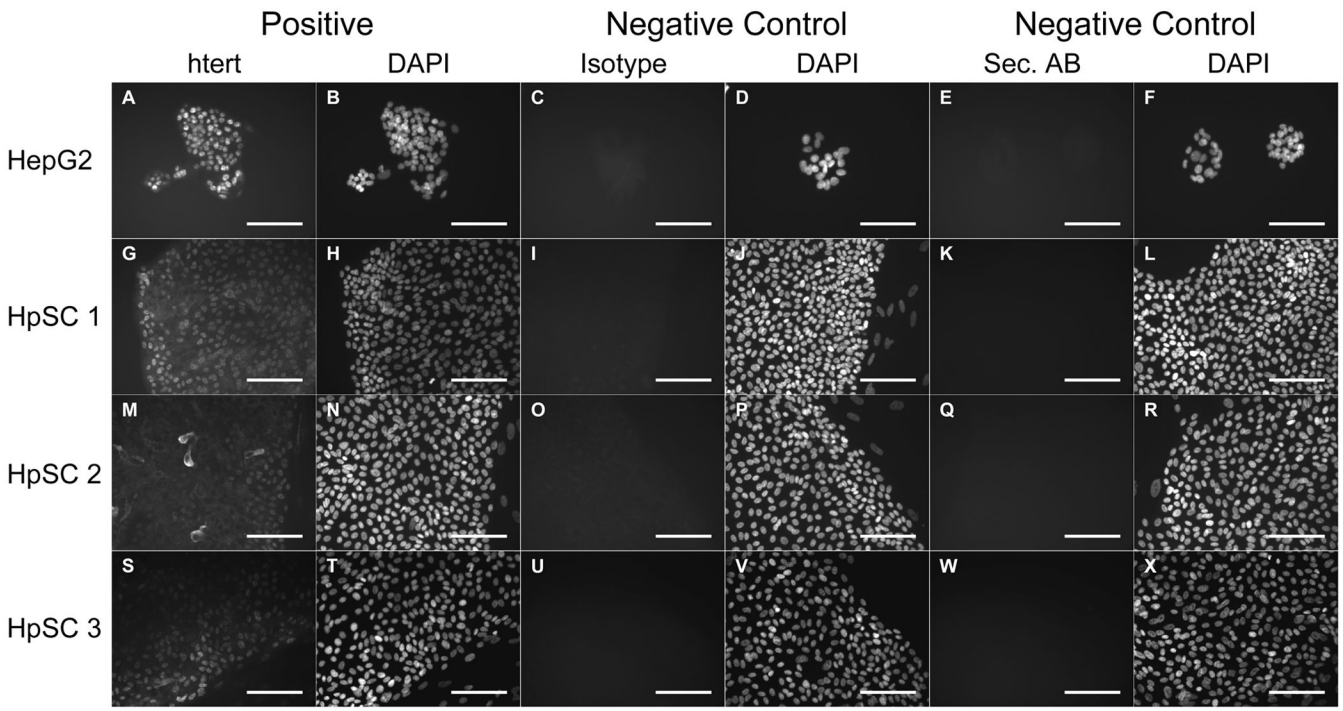

Figure 4. Immuncytochemistry for Telomerase

Colonies of hHpSCs from three different donors and HepG2 (positive control) were stained for telomerase enzyme. Isotype controls and controls with omission of primary antibody revealed no staining. In parallel, corresponding nuclei staining with DAPI were performed. Some hHpSCs at the perimeters of the colonies showed expression of telomerase, but cells in the center of the colonies usually were absent of any expression. Telomerase expression was observed in the nucleus. Occasionally, a few hHpSCs within a colony demonstrated cytoplasmic telomerase expression (M). Bars = 100 μm.

Table 1

PCR Conditions.

	Accession Number	Forward Primer Sequence (5’ to 3’)	Reverse Primer Sequence (5’ to 3’)	Product Length (bp)	PCR Conditions	Reference
hTR	NR_001566	acc taa ct gaga agg gcg t	gcc agc agc tga cat ttt tt	142	94°C 1 min; 26 cycles 96°C 15s, 55°C 30s, 72°C 30s	(Heine et al., 1998)[a]
hTERT	NM_198253	agc cag tct cac ctt caa ccg c	gga gta gca gag gga ggc cg	272	94°C 1 min; 35 cycles 94°C 30s, 63°C 30s, 72°C 30s	(Yen et al., 2007)[b]
GAPDH	NM_002046	tgt tgc cat caa tga ccc ctt	ctc cac gac gta ctc agc g	210	94°C 1 min; 25 cycles 94°C 30s, 58°C 30s, 72° 30s	(Harvard Primer Bank, ID 7669492a2)[c]

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[b]Yen, M.L., C.C. Chien, I.M. Chiu, H.I. Huang, Y.C. Chen, H.I. Hu, and B.L. Yen. 2007. Multilineage differentiation and characterization of the human fetal osteoblastic 1.19 cell line: a possible in vitro model of human mesenchymal progenitors. Stem Cells. 25:125–31.

[c]Wang, X, and Seed B. 2003. A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res. 31:e154.