

Telomerase Activation in Liver Regeneration and Hepatocarcinogenesis: Dr. Jekyll or Mr. Hyde?

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Abstract: The liver has a remarkable capability to restore its functional capacity following liver injury. According to the current paradigm, differentiated and usually quiescent hepatocytes are the primary cell type responsible for liver repair. As reserve compartment, bipotent hepatic progenitor cells are activated, especially if extensive loss or damage of hepatocytes with impaired replication occurs, e.g. in cirrhotic liver tissue. Recently, animal studies have suggested that liver regeneration following partial hepatectomy is associated with telomerase activation. Telomerase, a ribonucleoprotein with reverse transcriptase activity, plays a pivotal role in maintaining telomere length and chromosomal stability in proliferating cells. In cells lacking telomerase activity, replication-associated telomere shortening limits the replicative lifespan. Therefore, in the context of liver regeneration, telomerase activation might be a cellular mechanism to confer an extended lifespan to replicating hepatocytes and hepatic progenitor cells. On the other hand, high levels of telomerase activity are a hallmark of cancer, including hepatocellular carcinoma. Moreover, recent data indicate that telomerase activation may be an early event in hepatocarcinogenesis. At present, it is unclear, whether telomerase activation preserves the non-malignant phenotype and replicative longevity of liver cells or constitutes an early alteration obligatory for an unlimited proliferation and malignant transformation.

Keywords: Hepatic progenitor cells, hepatocellular carcinoma, liver-directed cell therapies, liver regeneration, telomere length, telomerase activation.

INTRODUCTION

The ribonucleoprotein telomerase plays a pivotal role in maintaining telomere length and chromosomal stability in proliferating eukaryotic cells. In contrast to most quiescent and differentiated human cells, including hepatocytes, high levels of telomerase activity have been detected in stem cells and certain highly proliferative cell types, such as activated lymphocytes. In cell culture, proliferating telomerase-negative human cells lose telomeric sequences until the cells exit the cell cycle with critically short telomeres. Telomere maintenance, mostly through telomerase activation, has been proposed as an obligatory step in hepatocarcinogenesis. Based on studies in telomerase knockout mice with critically short telomeres, divergent functions have been suggested for telomerase and telomere stability in cancer initiation and progression. For example, absence of telomerase activity and telomere dysfunction may promote genetic instability with an increased risk for mutations in the initial stages of hepatocarcinogenesis. On the other hand, recent studies identified telomerase activation in liver regeneration and early in hepatocarcinogenesis. In liver regeneration, telomerase activation may be crucial for genetic stability and replicative longevity of hepatocytes and hepatic progenitor cells. Telomerase activation allows an unlimited cellular proliferation, which clearly is also a hallmark of cancer. In

this review, we attempt to integrate novel data describing telomerase activation in liver regeneration and tumor initiation with the complex role of telomere dysfunction in hepatocarcinogenesis.

TELOMERE BIOLOGY AND TELOMERASE ACTIVATION IN CELLULAR REPLICATION

The physical ends of mammalian chromosomes are formed by tens of kilo bases of telomeric repeats, tandem arrays with the sequence (TTAGGG)_n [1]. Telomeres are predominantly double-stranded with a single-stranded 3'-protrusion of a few hundred nucleotides. By looping of the double-stranded DNA sequence and sequestering of the 3'-overhang, telomeres form molecular caps to protect chromosome ends against degradation and activation of DNA-damage response pathways [2]. Various proteins stabilize the telomeric loop structure, including telomeric repeat-binding factor (TRF) 1 and 2, and TRF1-interacting nuclear protein 2 (TIN2) [3]. With each cell division, telomeric repeats are lost and telomeres progressively shorten, for example, due to the end-replication problem [4]. After a certain number of cell divisions, replication-associated telomere shortening renders telomeric caps unstable and the end of chromosomes unprotected. This results in a dramatic upsurge in chromosomal aberrations, in particular translocations and formation of dicentric chromosomes. Eventually, cells with unstable chromosome ends are recognized by the DNA damage response machinery as double strand breaks leading to cell cycle exit and

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replicative senescence, a post-mitotic, quiescent state [5,6]. If the senescence-associated DNA damage checkpoint is bypassed, for example by transfection of cultured cells with the simian virus 40 large-T oncoprotein, cells continue to grow for a limited number of additional cell doublings until chromosomal instability results in cell crises and death.

In contrast to most somatic cells, germ cells and tumor cells are capable of undergoing an infinite number of cell doublings [7]. Telomerase, first described by Greider and Blackburn [8] as the enzyme performing the *de novo* synthesis of telomeric repeats, is a unique ribonucleoprotein consisting of a telomerase RNA component (TERC) that contains the template sequence on which telomeric repeats are generated, and multiple proteins, most importantly the catalytic subunit telomerase reverse transcriptase (TERT) [2]. Normal human somatic cells, including quiescent hepatocytes [9] and aging human fibroblasts [10], have low or undetectable levels of telomerase activity, because the cells do not express the catalytic subunit TERT. TERT expression has been identified as rate-limiting for the level of telomerase activity. The *in vitro* replicative capability of telomerase-negative human hepatocytes is limited by progressive telomere shortening [9].

HEPATOCTES AND HEPATIC STEM CELLS IN LIVER REGENERATION

The human liver has a remarkable capability to restore its functional capacity in response to loss of liver parenchyma. For example, partial hepatectomy induces regeneration by compensatory hyperplasia, a process in which cells in the liver remnant expand to replace the lost liver function [11]. According to recent models, differentiated and normally quiescent hepatocytes are the primary cell type responsible for compensatory hyperplasia, especially following partial hepatectomy or administration of carbon tetrachloride or acetaminophen in rodent models [12,13]. Functionally, expansion of existing hepatocytes is the most efficient way to rapidly restore liver function. In addition, liver regeneration studies, e.g. serial hepatocyte transplantation in mice with permanent regenerative pressure, have demonstrated a remarkable replicative capacity for mature murine hepatocytes [14,15]. Liver regeneration driven by the replication of existing hepatocytes is controlled by multiple pathways organized into different networks involving cytokines, growth factors, and metabolic processes. During the last decade, important factors in these networks have been identified and studied, in particular tumor necrosis factor, interleukin-6, epidermal and hepatocyte growth factor, and their corresponding receptors [16]. As reserve compartment, bipotent hepatic progenitor cells are activated if extensive loss or damage of hepatocytes with an impaired replication capability occurs [17]. These cells, also known as hepatoblasts, reside in the most peripheral branches of the biliary tree, the canals of Hering. The canals of Hering have also been identified as niche for putative intrahepatic stem cells. The role of hepatoblasts and intrahepatic stem cells in liver regeneration, especially in cirrhosis with diminished potential for hepatocyte replication, is still not entirely clear. Extensive proliferation involving ductular hepatocytes, considered to be an intermediate cell type between hepatic progenitor cells and hepatocytes, has been observed after

massive hepatic necrosis in humans [18]. Moreover, proliferation of hepatic progenitor cells has been detected in small cell dysplastic foci and cirrhotic liver tissue [19]. However, this process does not lead to extensive regeneration and is essentially ineffective in restoring the normal liver parenchyma. In summary, it appears as if liver regeneration is sustained by an inverse stem cell system with progenitor cells being a reserve population instead of the primary compartment for organ repair. Because of their capability to regenerate the liver following liver injury, which is unique among differentiated cells in human tissue, hepatocytes have been portrayed as the most efficient "stem cell" in the liver [12].

Another area of considerable controversy concerns the contribution of extrahepatic stem cells, in particular hematopoietic and bone marrow derived mesenchymal stem cells, as origin of hepatocytes in liver regeneration. Rodent models and careful evaluation of human liver samples following liver or bone marrow transplantation have generated conflicting results. Moreover, fusion between transplanted hematopoietic cells and hepatocytes generating tetraploid cells rather than true transdifferentiation has been suggested as principal mechanism for the formation of hepatocytes from bone marrow cells [20,21]. In a recent review of 77 reports addressing the capability of hematopoietic cells to generate hepatocytes, the authors conclude that hematopoietic cells contribute little to hepatocyte generation under either physiological or pathological conditions [22]. Currently, there is no compelling evidence that extrahepatic stem cells play an important role in liver regeneration.

TELOMERASE ACTIVATION IN LIVER REGENERATION

Certain highly proliferative cell types, such as human B lymphocytes in the germinal center, are capable of expressing low or transiently high levels of telomerase activity upon commitment to clonal expansion to decrease the net rate of telomere loss and to confer an extended but still finite lifespan to these cells [23-26]. In line with this observation, recent data indicate that telomerase is actively regulated throughout the cell cycle in regenerating mouse liver tissue [27]. Unfortunately, there are significant differences between mice and humans regarding telomere biology that are possible concerns in the use of murine models to investigate telomere maintenance and telomerase regulation. For example, in marked contrast to humans, commonly employed inbred laboratory mouse strains have approximately ten times longer telomeres (up to 150 kilo bases) and express robust levels of telomerase activity in a wide range of normal somatic tissues, including normal liver [28]. Therefore, telomere attrition is not considered as a major cause for mouse-cell senescence [29]. According to current studies, these divergences may be attributed to different TERT promoter organization and activity [30].

To circumvent the concerns in the use of mouse models to investigate telomerase regulation, we employed a surgical pig liver regeneration model. Pigs display very low levels of telomerase activity in the liver and have telomeres about twice as long as those of humans [31]. We employed partial

hepatectomy (70 to 80%) with and without early hepatic decompression to induce compensatory hyperplasia, i.e. regeneration by mature and normally quiescent hepatocytes. Three days after surgery, the postoperative day with peak mitotic activity [32], histology revealed mitotic hepatocytes as marker for liver regeneration. Telomerase activity increased significantly following liver injury and correlated to the number of mitotic hepatocytes¹. Our data supports an observation by Kotoula *et al.*, who evaluated various human liver samples and reported high levels of TERT expression, the rate-limiting catalytic subunit of the telomerase holoenzyme, in large regenerative and precancerous nodules that arise in cirrhotic liver tissue. Employing *in situ* hybridization, TERT expression was specifically observed in regions with active replication, for example in periportal and periseptal hepatocytes [33]. Based on the data presented by Kotoula *et al.* and our own data in a large animal model, we speculate that telomerase activation during liver regeneration might be a physiological response to preserve chromosomal stability and replicative longevity of proliferating liver cells. Therefore, telomerase activation might be regarded as a “healthy” reaction of the regenerating liver tissue. To functionally study the regulation of human telomerase during liver regeneration, new transgenic mouse models employing the human TERT promoter in comparison to the endogenous mouse TERT promoter have been established and should be helpful [28,30].

Without telomerase activation, continuous liver regeneration induced by chronic liver injury results in progressive telomere shortening limiting the regenerative capacity of hepatocytes. In telomerase knockout mice with critically short telomeres, liver regeneration is impaired by inhibiting cell cycle re-entry of cells with critically short telomeres [34]. Along this line, late generation telomerase knockout mice with critically short telomeres show defects in homeostasis of highly proliferative organs [35]. Moreover, in humans telomere shortening has been demonstrated in various extrahepatic tissues during aging and in chronic diseases with elevated cell turnover, including liver cirrhosis. Therefore, regarding the divergent role of telomere biology in liver repair and hepatocarcinogenesis, telomerase activation in replicating liver cells may be considered as protective.

GENETIC AND MORPHOLOGICAL CHANGES IN HEPATOCARCINOGENESIS

Carcinogenesis occurs as a multistep evolutionary process involving the disruption of a relatively small number of cellular pathways. This was first described by Vogelstein *et al.* in colorectal carcinoma [36,37]. Strong evidence for this model comes also from a landmark *in vitro* study performed by Hahn *et al.*, who demonstrated that the ectopic expression of three defined genes, specifically the simian virus 40 large-T oncoprotein, which disrupts both the p53 and the retinoblastoma tumor suppressor pathway, an oncogenic allele of H-ras, and TERT, is sufficient to convert normal human epithelial precursors into cancer cells [38]. In the current view, the sequential accumulation of genetic and

epigenetic changes during malignant transformation drives the progression of normal cells through preneoplastic states into invasive cancers [39]. In general, malignant growth requires: self-sufficiency in growth signals, insensitivity to growth inhibitors, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [40]. Among these characteristics of malignant cells, it has become increasingly clear that the predominant event necessary for carcinogenesis is cellular immortality, i.e. an unrestricted proliferative potential [41]. As a prerequisite for immortality, telomere maintenance, either by activation of telomerase or alternative lengthening of telomeres (ALT), has been proposed [42,43]. ALT in the absence of telomerase, e.g. by means of homologous recombination and copy switching of telomeric sequences, has been described in various organisms and recently also in immortalized and transformed human cell lines [44].

A rise in the incidence and mortality rate of hepatocellular carcinoma (HCC), currently being the fifth most common malignancy in men and the eighth in women, has been reported in developed countries [45]. HCC almost exclusively develops in patients with cirrhosis, a condition characterized by hepatocyte turnover and oxidative stress, and is currently the major cause of liver-related death in patients with compensated cirrhosis [45]. The major etiologies underlying HCC, including chronic hepatitis B and C, alcohol-related cirrhosis, metabolic liver disorders, and non-alcoholic fatty liver disease, act predominantly through the pathway of liver regeneration and cirrhosis, eventually resulting in malignant transformation of proliferating hepatocytes. Thus, malignant transformation of hepatocytes occurs regardless of the etiological agent mostly through a pathway of increased hepatocyte turnover and chronic liver injury. On the other hand, there is evidence that certain hepatotropic viruses, especially hepatitis B virus, play an additional direct role in the induction of HCC. For instance, insertional mutagenesis and transactivation of cellular genes by the hepatitis B virus X protein have been discussed [46]. While the molecular events driving the development of other malignancies, for example colorectal cancer and Bcr-Abl positive chronic leukemia, have largely been identified, the molecular pathogenesis of HCC is still not well understood. Several studies have shown chromosomal abnormalities in HCC compared to normal liver samples, particularly loss of heterozygosity on chromosome 1, 2q, 4, 5q, 6q, 8, 9, 10q, 11p, 13q, 14q, 16, 17, and 22q [46]. Loss of heterozygosity is characterized by a hemizygous genotype in specific chromosomal regions due to deletions or other mutational events. In these regions, only one allele is present which increases the risk of tumor formation if the remaining allele is inactivated by a point mutation and contains regulatory elements for the cell cycle, e.g. a tumor suppressor gene.

On the morphological level, continuous regeneration of hepatocytes as a consequence of chronic liver injury and oxidative stress results in cytological and histological changes with features suggestive of precancerous phenotypes. A variety of putative precancerous lesions within the hepatic parenchyma in cirrhosis representing different stages of tumor development have been described. Based on the growing number of publications on the morphological changes in cirrhotic livers, the International Working Party

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for Nodular Hepatocellular Lesions, sponsored by the World Congress of Gastroenterology in 1994, has introduced a common nomenclature [47]. According to a recent refinement of this nomenclature, dysplastic liver nodules, which occur with increased frequency in cirrhotic livers with HCC, can be separated into high grade dysplastic nodules and low grade dysplastic nodules. High grade dysplastic nodules, also called adenomatous hyperplasia, and low grade dysplastic lesions are both considered as precancerous changes [48]. The precancerous nature of dysplastic nodules has been established by clinicopathological studies that have demonstrated evolution of dysplastic nodules to HCC within months to a few years of follow-up [49]. Based on these studies, the morphological changes during human hepatocarcinogenesis have been recognized and defined.

TELOMERASE ACTIVATION IN HEPATOCARCINOGENESIS

During the last decade, key genes and pathways involved in the development of subgroups of HCC have been detected, including alterations in receptor tyrosine kinase and ubiquitin-proteasome pathways [41], activation of the Wnt pathway, either by gain-of-function mutations in the β -catenin gene or loss-of-function mutations in AXIN1 and AXIN2 [50], and mutations in the retinoblastoma tumor suppressor pathway or in p16^{INK4} [46]. Furthermore,

upregulated expression of various growth factors, such as insulin-like growth factor II, insulin receptor substrate 1, transforming growth factor α and β , and hepatocyte growth factor, and of components of their signaling pathways, have been suggested to play a role in hepatocarcinogenesis [46]. Overexpression of the mammalian target of rapamycin (mTOR) subfamily of proteins, which plays a role in the PI3K/Akt signal-transduction pathway, was also reported in HCC [51]. Genetic alterations and dysregulation of growth factor signaling in human HCC have recently been reviewed [52,53]. Despite the number of studies investigating molecular events related to hepatocarcinogenesis, none of the above-summarized alterations has been observed consistently in the majority of cases of HCC. Therefore, no convincing model of oncogene activation or tumor suppressor inhibition has emerged so far. In sharp contrast, almost all cases of HCC exhibit activation of telomerase, emphasizing the role of an unconstrained cell proliferation for the development of cancer. In large series, less than 20% of HCC lacked detectable telomerase activity [54,55].

In 1997, Kojima *et al.* confirmed high levels of telomerase activity in 85% of human HCC. In contrast to the consistently detected high levels of telomerase activity, telomere length showed a wide distribution in HCC ranging from 2.0 to 16.0 kilo bases with telomeres considerably shorter than the surrounding nontumorous tissue in 44% and longer in 17% [54]. In our own investigation, we detected

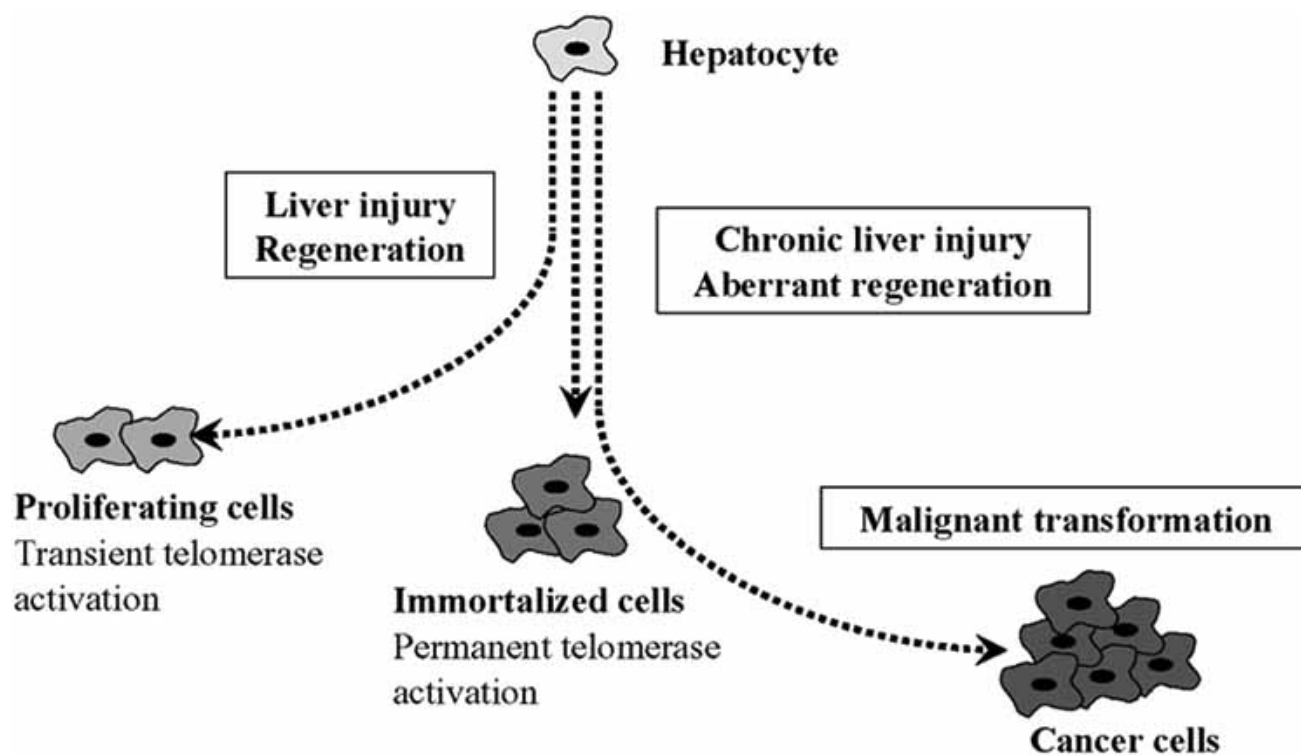


Fig. (1). Schematic representation of the putative role of telomere biology in liver regeneration and hepatocarcinogenesis. Telomerase is transiently activated in proliferating liver cells to provide chromosomal stability and replicative longevity. Permanent telomerase activation induced by chronic liver injury represents an aberrant regeneration and permits unlimited proliferation of replicating liver cells and might, together with additional genetic changes, lead to malignant transformation.

very low telomerase activity in normal liver tissue and liver adenoma, whereas high levels of telomerase activity were measured in liver metastases and in 7 out of 9 HCC samples (78%), with a tendency for higher levels in poorly-differentiated compared to moderately-differentiated tumors (H. Wege *et al.*, unpublished data). Various groups have demonstrated telomerase activation in precancerous hepatic nodules. For example, Oh *et al.* have observed shortening of telomeres and activation of telomerase in dysplastic nodules, considered to be a precancerous lesion in multistep hepatocarcinogenesis, with a significant change in the transition from low grade dysplastic nodules to high grade dysplasia [56]. Furthermore, Hytioglou *et al.* reported that telomerase activity was positive or strongly positive in 86% of regenerative nodules and low grade dysplastic nodules [57]. In addition, Takaishi *et al.* demonstrated higher telomerase activity levels in precancerous hepatic nodules compared to nontumorous chronic liver diseases [58]. Recently, a marked increase in the expression of TRF1, TRF2, and TIN2 in high grade dysplastic nodules was demonstrated [59]. All genes showed a significant negative correlation with telomere length and thus might play a role in hepatocarcinogenesis. Based on these observations, telomerase activation may be considered as early event in the multistep development of HCC. Regarding later stages of hepatocarcinogenesis, for example tumor progression, Farazi and co-workers showed that telomere dysfunction suppressed the growth of established tumors in telomerase knockout mice with critically short telomeres [60]. Moreover, a variety of studies have shown that telomerase-inhibitors limit the growth of telomerase-positive human cancer cell lines. Taken together, telomerase activation may be considered as rate-limiting step in tumor initiation and progression in human hepatocarcinogenesis. Therefore, early telomerase activation in replicating liver cells might represent a crucial step for malignant transformation, and hence, for an aberrant regeneration (Fig. 1).

In a different model, telomere shortening in telomerase-negative cells results in an increased genetic instability and is thus instrumental in tumor initiation. This model has been studied by Rudolph and co-workers [61-62] in the telomerase knockout mouse lacking the RNA component of telomerase (mTERC^{-/-}). In these mice, critically short telomeres are observed in the sixth generation and are associated with a 4- to 6-fold increase in spontaneous tumor incidence. A possible explanation for this increase in tumor formation is the increase in genetic instability potentially associated with critically short telomeres. In support of this hypothesis, p53 deficiency increases the frequency of chromosome fusions and aneuploidy and decreases apoptosis in late generation telomerase knockout mice [63]. Despite these insights, it has not yet been definitively established that telomere shortening contributes to genetic instability in human hepatocarcinogenesis. Differences in telomere length, cell culture profile, and tumor spectrum in mice and humans have contributed to the continued ambiguity of the role of telomeres in human hepatocarcinogenesis. Difference between mice and humans are not solely an effect of the much longer telomere length in mice compared to humans, but are also due to the absence of a stringent senescence checkpoint in mice [64]. For instance, it has been demonstrated that mouse cells with very short telomeres

bypass senescence and immortalize spontaneously with kinetics similar to mouse cells with long telomeres [29]. In summary, telomerase activation with or without telomere dysfunction seems to be crucial in hepatocarcinogenesis. Therefore, telomerase activation in replicating hepatocytes and hepatic progenitor cells might be associated with an increased risk for malignant transformation and may represent a possible link between regeneration, immortalization, and malignant transformation.

TELOMERASE-IMMORTALIZED CELLS FOR LIVER-DIRECTED CELL THERAPIES

Metabolic liver diseases constitute an important health problem. Potentially, hepatocyte transplantation offers an alternative to liver transplantation [65,66], which, as the only currently available definitive treatment for inborn metabolic liver disorders, is restricted by the shortage of donor organs [67]. Although the use of primary human hepatocytes would be ideal for liver-directed cell therapies, the availability of these cells is limited and the expansion of human hepatocytes in culture is difficult [68]. Using malignant cell lines, such as HepG2 or C3A cells, or xenogenic hepatocytes for liver-directed cell therapies carries potential risks to recipients, for example inoculation of tumor cells or transmission of animal viruses, notably porcine endogenous retrovirus [69]. This situation has prompted intensive research to generate human hepatocyte-derived cell lines and to explore various strategies for immortalizing and expanding the number of human hepatocytes for the purpose of tissue engineering.

It has been demonstrated in various human cell types [70-72] that the ectopic expression of TERT reconstitutes telomerase activity resulting in an extended proliferative lifespan of cells. Moreover, TERT-immortalized cells retained the biological features of their normal counterparts and responded to various kinds of DNA damage with cell cycle arrest [73,74]. We immortalized proliferating human hepatocytes using an approach based on the observation that telomere-dependent replicative aging restricts the expansion of human hepatocytes in cell culture [9]. In addition, it was reported that cells from very young human donors, such as fetal and neonatal hepatocytes, display mitogen-independent spontaneous proliferation in cell culture, which decreases over several months [75]. These cells were thus very likely to be amenable to telomerase-mediated immortalization. Following successful TERT-delivery with a retroviral vector, fetal and neonatal human hepatocytes exhibited abundant transgene expression with high levels of telomerase activity and telomere maintenance. Telomerase-immortalized fetal human hepatocytes proliferated beyond senescence for more than 350 population doublings (end of the experiment). Markers of hepatocellular functions were detected in immortalized clones at the mRNA and protein level as well as in functional assays. Intrahepatic cell transplantation studies in immunodeficient mice showed integration and differentiation of telomerase-immortalized fetal human hepatocytes in the hepatic parenchyma [76]. Following telomerase-reconstitution, the derived clones displayed no evidence of a transformed phenotype (Fig. 2). Furthermore, cytogenetic studies revealed a diploid karyotype (46,XX) in parental fetal cells during the initial passages and following

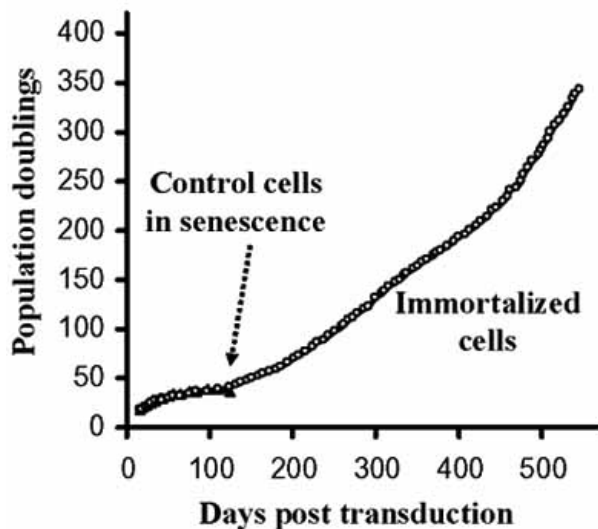


Fig. (2). Absence of cancer traits in telomerase-immortalized human hepatocytes. The growth curves on the left demonstrate proliferation of telomerase-reconstituted fetal human hepatocytes beyond senescence (immortalized cells), observed in telomerase-negative cells after 35 to 40 population doublings (control cells). Various *in vitro* and *in vivo* assays, summarized in the table on the right, confirmed an untransformed phenotype for telomerase-immortalized fetal human hepatocytes.

telomerase reconstitution and proliferation beyond senescence.

In animal models, telomerase-immortalized hepatocytes have already successfully been used to treat liver diseases. In a recently published study, primary human hepatocytes were immortalized employing lentiviral vectors coding for the simian virus large-T oncoprotein, telomerase, and Bmi-1, a downregulator of the p16 and p19^{Arf} tumor suppressor genes. The immortalized hepatocytes grew continuously yet were non-tumorigenic, and conserved defining properties of primary hepatocytes, including the ability to secrete liver-specific proteins and to detoxify drugs. Implantation of encapsulated immortalized human hepatocytes rescued mice from lethal doses of acetaminophen [77]. In an attempt to create cells for the treatment of type 1 diabetes, telomerase-immortalized human fetal hepatocytes were transduced with the pancreatic duodenal homeobox 1 (Pdx1) gene, which plays key roles in pancreas development and is expressed in mature beta cells as well. Immortalized fetal human hepatocytes expressing Pdx1 activated multiple beta-cell genes, produced and stored considerable amounts of insulin, and released insulin in a regulated manner in response to glucose. Moreover, when transplanted into hyperglycemic immunodeficient mice, the cells restored and maintained euglycemia for prolonged periods [78]. In a similar experiment, telomerase-immortalized human hepatocytes were transfected with a modified insulin cDNA under the control of the L-type pyruvate kinase promoter. Xenotransplantation of the insulin-producing cells *via* the portal vein into immunosuppressed, totally pancreatectomized pigs decreased hyperglycemia and prolonged survival [79].

In summary, generation of immortalized human hepatocytes with preserved functional properties, particularly protein synthesis and enzymatic activities, with or without additional genetic modifications to produce cell lines with

Cancer traits	Normal	Control	Immortalized	Cancer
Contact inhibition	+	+	+	-
Serum-dependence	+	+	+	-
Damage response	+	+	+	-
Colony growth	-	-	-	+
Tumor formation	-	-	-	+

desired phenotypic features, such as insulin secretion in response to hyperglycemia, constitutes a promising approach to establish novel cell sources for cell therapies. To this regard, telomerase-mediated immortalization is especially interesting because telomerase activation does not disrupt the differentiation potential of progenitor cells and by itself does not induce malignant transformation [76]. Other groups have also reported that ectopic TERT expression is not associated with neoplastic transformation [70,72,73]. However, as summarized above, telomerase activation has been suggested as early event during hepatocarcinogenesis permitting an unrestricted proliferation. Moreover, long-term culture in other telomerase-immortalized cell lines have demonstrated that telomere stabilization *via* telomerase reconstitution does not entirely protect against karyotype abnormalities [80,81]. For example, Mondello *et al.* reported karyotype instability and anchorage-independent growth in telomerase-immortalized human fibroblasts from two centenarian individuals [80]. This study underscores the possible hazards in using cells with an unrestricted proliferative potential for cell therapies in humans. Additional studies are definitely required to evaluate the genetic stability in telomerase-immortalized human cells under the influence of oxidative stress and to investigate the potential long-term risks following engraftment of telomerase-immortalized human cells in immunosuppressed mice.

In an alternative approach, embryonic stem cells with unlimited proliferative potential may be employed to generate hepatocytes, for example, by applying culture conditions that induce hepatocyte-specific differentiation [68]. Along this line, we investigated various combinations of culture media, growth and differentiation factors, and extracellular matrix components [82]. The optimal culture condition, consisting of Iscove's modified Dulbecco's medium with 20% fetal bovine serum, human insulin, dexamethasone, and collagen type I precoating, directed

mouse and human embryonic stem cells along a hepatocyte lineage with albumin expression 7% as high as in primary mouse hepatocytes and 1% as high as in primary human hepatocytes. Albumin was also detected by immunoblotting and immunocytochemistry. Moreover, using an albumin promoter construct with green fluorescent protein as detectable marker albumin-expressing cells were amenable to selection, subcloning, and further expansion in culture [82]. Various alternative methodologies for the differentiation of embryonic stem cells using either spontaneous or directed differentiation have recently been reviewed [83]. Differentiated embryonic stem cells have thus far not been employed to treat patients with liver failure and insufficient liver regeneration. Certainly, this area has great potential for cell-based therapeutics; however, additional work is necessary to fully understand the involved pathways.

CONCLUSION

While telomerase activation seems to be required for tumor growth, telomerase activation during liver regeneration may facilitate genomic stability thus preventing tumor initiation. Therefore, telomerase activation appears to have both tumor suppressive and oncogenic roles during liver regeneration and hepatocarcinogenesis. Immortalizing cells by telomerase reconstitution offers great possibilities for tissue engineering and cell therapies. However, further studies are necessary to elucidate the risk for malignant transformation in cells with stable telomeres and unrestricted proliferative potential. These studies should help to reveal the role of telomerase in immortalized cells and to uncover whether telomerase activation acts as Dr. Jekyll or Mr. Hyde or both, depending on other yet unknown co-factors, in liver regeneration and hepatocarcinogenesis.

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