Review

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Strategies for immortalization of primary hepatocytes

Eva Ramboer[1,*], Bram De Craene[2,3], Joery De Kock[1], Tamara Vanhaecke[1], Geert Berx[2,3], Vera Rogiers[1], Mathieu Vinken[1]

[1]*Department of Toxicology, Center for Pharmaceutical Research, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussel, Belgium;*
[2]*Unit of Molecular and Cellular Oncology, Inflammation Research Center, VIB, Technologiepark 927, 9052 Zwijnaarde, Belgium;*
[3]*Department of Biomedical Molecular Biology, Ghent University, 9052 Ghent, Belgium*

Summary

The liver has the unique capacity to regenerate in response to a damaging event. Liver regeneration is hereby largely driven by hepatocyte proliferation, which in turn relies on cell cycling. The hepatocyte cell cycle is a complex process that is tightly regulated by several well-established mechanisms. *In vitro*, isolated hepatocytes do not longer retain this proliferative capacity. However, *in vitro* cell growth can be boosted by immortalization of hepatocytes. Well-defined immortalization genes can be artificially overexpressed in hepatocytes or the cells can be conditionally immortalized leading to controlled cell proliferation. This paper discusses the current immortalization techniques and provides a state-of-the-art overview of the actually available immortalized hepatocyte-derived cell lines and their applications.

Introduction

In the field of hepatology, when orthotopic liver transplantation is not possible, human primary hepatocytes represent the 'gold standard', in particular for the establishment of bioartificial liver (BAL) support systems [1,2]. They also serve as an important tool in research and are of particular interest for *in vitro* pharmacotoxicology [3,4]. Consequently, there is a considerable and increasing demand for human primary hepatocytes, yet their use is hampered by inadequate supply, high cost, high variability and low *in vitro* proliferation capacity. These constraints have prompted a large-scale search for alternative cell sources, such as hepatic cell lines and stem-cell derived hepatocytes [2,5–9]. In contrast to primary cells, cell lines are readily available, and usually have an unlimited growth potential and high reproducibility [10,11]. Hepatic cell lines are either derived directly from liver tumor tissue or artificially generated from primary hepatocytes *in vitro* [5,6]. Several hepatoma-derived cell lines preserve some liver-specific functions, but most of them, with exception of the HepaRG® cells, do not exhibit sufficient functionality to be of pharmaco-toxicological relevance [12–18]. Immortalized hepatocytes are typically derived from healthy primary hepatocytes by using a defined immortalization strategy. Both fetal and adult hepatocytes from different species have already been successfully immortalized, whether or not using a combination of viral oncogenes and the human telomerase reverse transcriptase (hTERT) protein [7,9,19–25]. The purpose of this paper is to discuss the different current immortalization strategies and to provide an overview of the actually available immortalized hepatic cell lines and their applications. To fully understand these immortalization techniques, the processes of hepatocyte proliferation and senescence are briefly outlined in the preceding part.

Key Points

Hepatocyte immortalization strategies

- Commonly used immortalization genes include viral oncogenes and *hTERT*
- Gene transfer is accomplished by viral and non-viral methods
- Conditional immortalization enables the production of growth-controlled cell lines

Keywords: Hepatocyte; Proliferation; Senescence; Immortalization.
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* Corresponding author. Address: Department of Toxicology (FAFY), Center for Pharmaceutical Research (CePhaR), Faculty of Medicine and Pharmacy, Vrije Universiteit Brussel (VUB), Laarbeeklaan 103, B-1090 Brussels, Belgium. Tel.: +32 2 477 45 87; fax: +32 2 477 45 82.
E-mail address: eramboer@vub.ac.be (E. Ramboer).

Abbreviations: BAL, bio-artificial liver; (h)TERT, (human) telomerase reverse transcriptase; G, gap; DNA, deoxyribonucleic acid; mRNA, messenger ribonucleic acid; MAPK, mitogen-activated protein kinase; pRB, retinoblastoma protein; HGF, hepatocyte growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; cdk(s), cyclin-dependent kinase(s); S, synthesis; M, mitosis; cdki, cdk inhibitor; PH, partial hepatectomy; SV40 Tag, simian virus 40 large T antigen; HPV16, human papillomavirus type 16; Mo-MLV, Moloney Murine Leukemia virus; BMI-1, B lymphoma Mo-MLV insertion region 1 homolog; HSV-TK, herpes simplex virus thymidine kinase; tTA, tetracycline transactivator; rtTA, reverse tetracycline transactivator; HIV, human immunodeficiency virus; HAC, human artificial chromosome; ALF, acute liver failure.

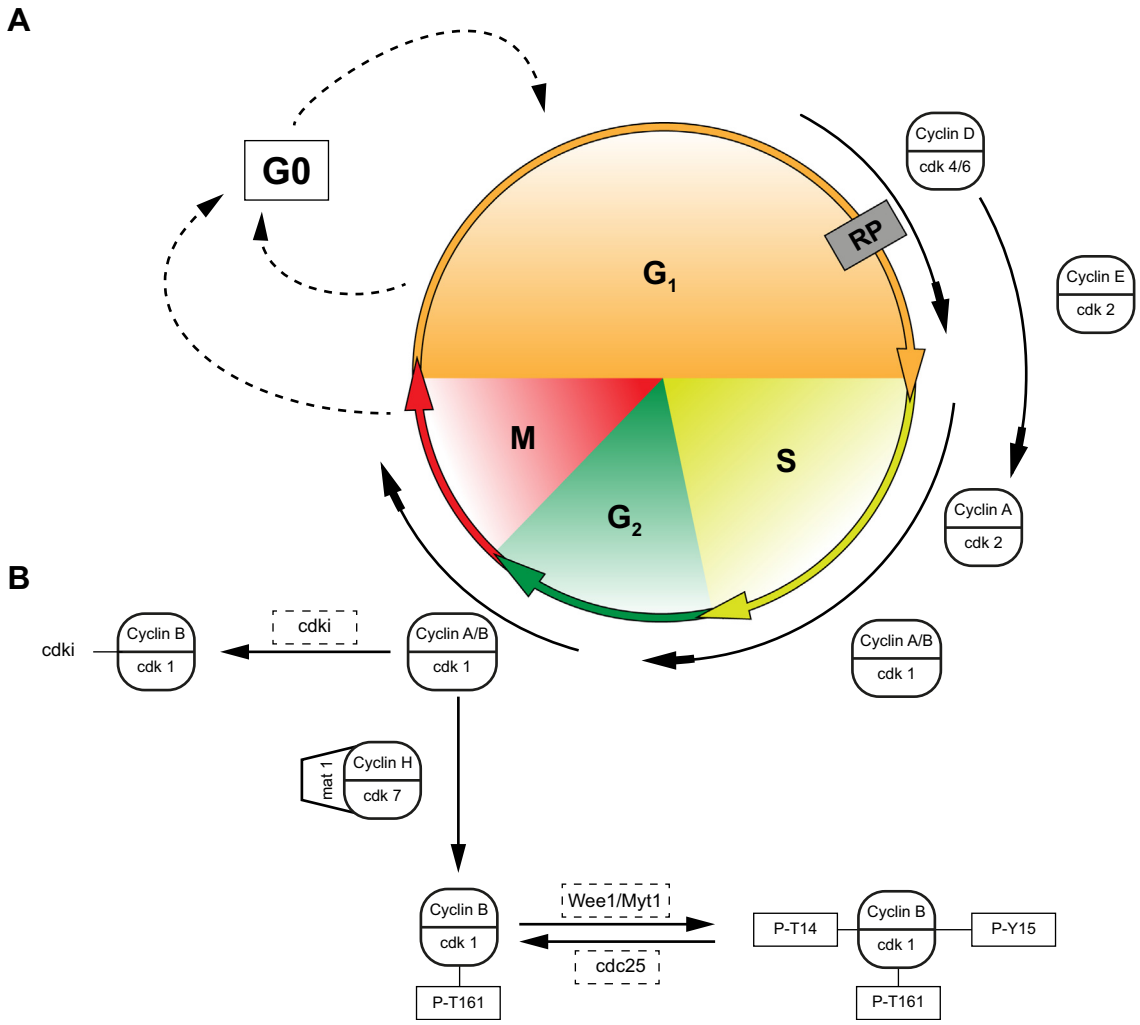

Fig. 1. The hepatocyte cell cycle and its regulation. (A) The hepatocyte cell cycle, as in other eukaryotic cells, is composed of four phases namely the G1, S, G2, and M phase. Under physiological conditions, most hepatocytes in the adult liver escape the active cell cycle and enter a quiescent stage, known as the G0 phase. In this state, hepatocytes do not proliferate, but remain metabolically active. Upon appropriate stimulation, hepatocytes re-enter the cell cycle in the G1 phase [42,174]. Progression through the mid-late G1 phase is growth factor-dependent. Once beyond the mitogen-dependent restriction point (RP), the cell cycle is completed autonomously, driven by the sequential activation of a series of structurally related serine/threonine protein kinases, the cyclin dependent kinases (cdk) [42]. In contrast with other mammalian cells, hepatocytes possess active cyclin A-cdk1 and cyclin B-cdk1 complexes during the S-phase of their cell cycle, which is suggested to allow rapid and efficient hepatocyte proliferation [175]. (B) The kinase activity of the cdks is tightly regulated by several different mechanisms, including binding to cyclins, binding by cdk inhibitors (cdki) and various phosphorylation/dephosphorylation events. For example full activation of the cyclin B-cdk1 complex requires its phosphorylation (P) on threonine 161 by the cdk-activating kinase (CAK). Other phosphorylation/dephosphorylation events fine-tune kinase activity and thereby facilitate proper mitotic action. The kinases Myt1 and Wee1 negatively affect kinase activity by phosphorylating cdk1 on threonine 14 (T14) and tyrosine 15 (Y15), whereas cdc25 phosphatase restores kinase activity by dephosphorylation of the same amino acids. Furthermore, Cip/Kip cdki can bind to the cyclin B/ckd1 complexes and inhibit their action [39,42,51,55]. Adapted from [28,42,51]. CAK, cdk-activating kinase; cdk(i), cyclin dependent kinase (inhibitor); G, gap; M, mitosis; P, phosphorylated; RP, restriction point; S, synthesis; T, threonine; Y, tyrosine.

Hepatocyte proliferation

Priming phase and commitment to hepatocyte cell cycle progression

Under normal conditions, the adult liver has very little proliferative activity. However, upon partial removal of liver tissue, the remaining intact hepatic lobes start to grow and liver mass is restored within seven to ten days due to the proliferation of mature hepatocytes [26,27]. Multiple genes involved in cytokine networks become differentially expressed and regulate the initiation of this liver regeneration, a process called the "priming phase" [28–30]. During this step, G0/G1 cell cycle transition and early G1 progression are accomplished and hepatocytes become responsive to mitogenic signals, which leads to deoxyribonucleic acid (DNA) replication [28,30,31]. During collagenase perfusion of the liver, a critical step in the isolation procedure of hepatocytes, the messenger ribonucleic acid (mRNA) levels of the proto-oncogenes *c-Jun* and *c-Fos* rapidly increase, suggesting that enzymatic liver dissociation triggers the G0/G1 cell cycle transition of hepatocytes [32,33]. Indeed, collagenase perfusion of the liver, which is accompanied by release of the cytokine tumor necrosis factor α as well as activation of the intracellular nuclear factor kappa-light-chain-enhancer of activated B cells and mitogen-activated protein kinase (MAPK) pathways, can

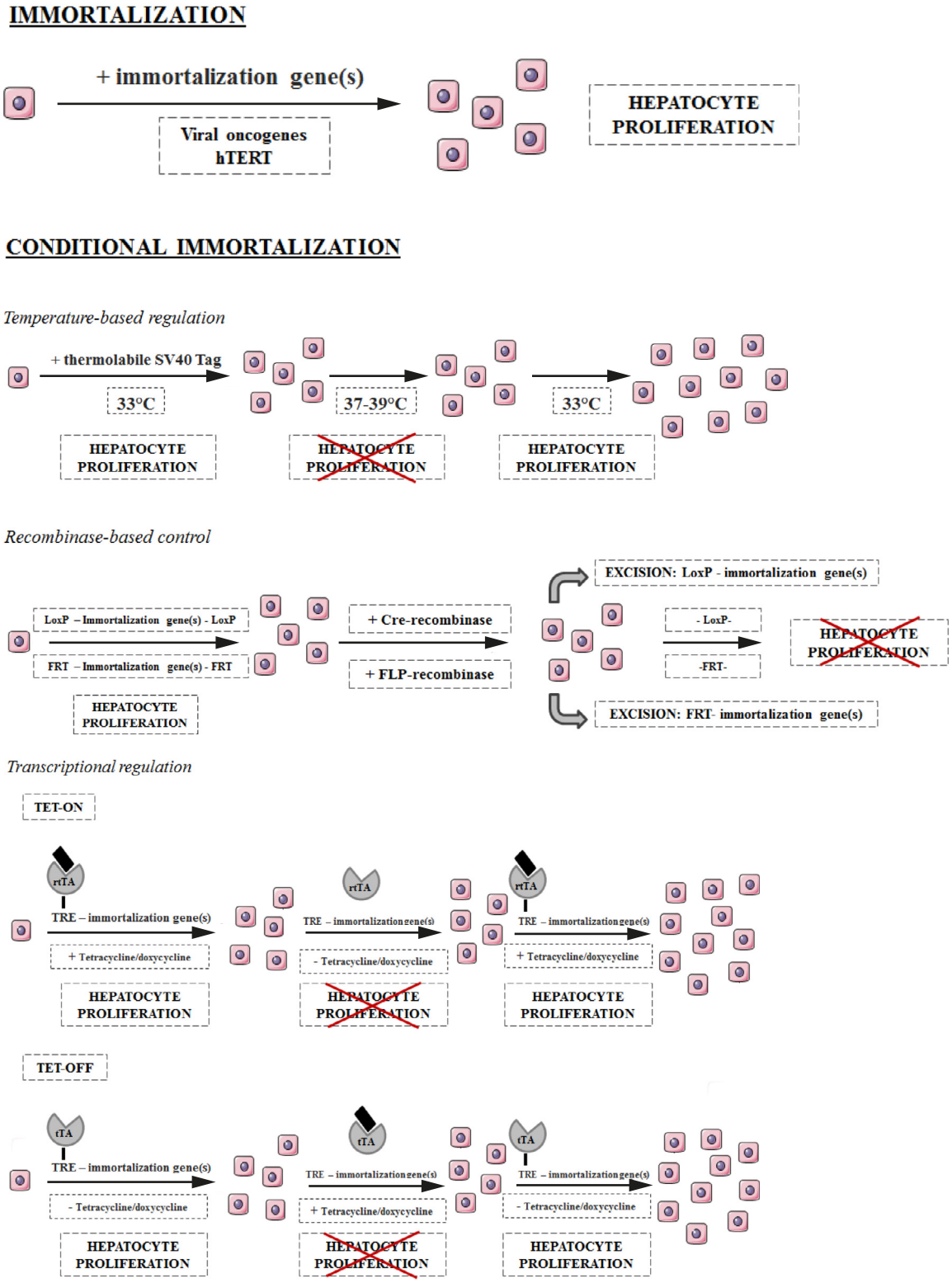

Fig. 2. Hepatocyte immortalization strategies. Several hepatocyte immortalization strategies are available, including transduction or transfection of prototypical immortalization genes. Conditional immortalization by temperature-based regulation, recombinase-based control and transcriptional regulation have been introduced to establish growth-controlled cell lines. Adapted from [10]. rtTA, reverse tetracycline transactivator; TRE, tetracycline responsive element; tTA, tetracycline transactivator.

induce priming of quiescent hepatocytes [28,32,34–36]. When the freshly isolated hepatocytes are plated, the sequentially increased expression of other proto-oncogenes, such as JunB, JunD, *c*-Myc, p53 and *c*-Ki-ras, indicates that the hepatocytes can proceed to the mid-late G1 phase [28,32]. However, further progression towards the G1/S cell cycle boundary is only possible after stimulation with appropriate growth factors to overcome the mitogen-dependent mid-late G1 restriction point [32]. This major checkpoint is regulated by the tumor suppressor retinoblastoma protein (pRB) and controls whether the cellular environment supports proliferation [37–39]. The need for mitogenic signals to pursue cell cycling has also been shown *in vivo*, though intrinsic differences exist between the *in vivo* and *in vitro* conditions [31,40]. *In vivo*, normal adult hepatocytes return to the G0 state in the absence of growth factor stimulation, but that is not the case *in vitro* [26,36,40]. After attaching to the culture dish, surviving cells remain at the mid-late G1 restriction point, do not proliferate and die early [36,41].

Several studies designated cyclin D1 as the major intracellular mediator of the mitogenic signals responsible for the regulation of hepatocyte proliferation [32,40,42–45]. As such, overexpression of D-type cyclins seems sufficient to overcome the mid-late G1 restriction point and trigger hepatocyte proliferation both, *in vivo* and *in vitro*, in the absence of mitogens [43,45,46]. Though, the latter has been challenged by Wierod *et al.* [47]. Interestingly, fetal hepatocytes, which express both cyclin D2 and D3, possess a high proliferation rate that is, at least partly, independent of mitogenic pathways and characterized by the constitutive phosphorylation of pRB [48,49].

Critical growth factors involved in hepatocyte cell cycling include hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor (TGF) α, heparin-binding EGF-like growth factor and amphiregulin [29,50]. Once past the mid-late G1 restriction point, hepatocytes are irreversibly committed to replicate and no longer require growth factors to complete the first cycle of cell proliferation [40,42]. From this point onwards, progression through the cell cycle proceeds autonomously and is driven by the sequential formation, activation and destruction of a series of structurally related serine/threonine protein kinase complexes, each composed of a regulatory and a catalytic subunit, cyclin and cyclin-dependent kinase (cdk), respectively [28,42].

Hepatocyte cell cycle regulation and control

To date, at least 20 different cdk proteins and 30 cyclins have been identified in mammalian cells. However, only some are involved in cell cycle regulation [28,51,52]. Whereas the cdks are expressed throughout the hepatocyte cell cycle, with the notable exception of cdk1, most cyclins display a temporal expression profile, leading to periodic activation of their respective cdk counterparts [36,42,53]. Since these individual cyclin/cdk complexes perform unique functions in the cell cycle, their sequential assembly and activation dictates the order in which the cell cycle events occur [28,51,54,55] (Fig. 1). Nevertheless, subsequent progression through the S, G2 and M phases can be impeded by additional cell cycle checkpoints, which are switched on in response to unfavourable conditions [42]. In this context, checkpoints at the G1/S and G2/M boundaries ensure the orderly unfolding of different cell cycle events and inhibit cell cycling in response to DNA damage. Overall, mechanisms associated with activation of the p53/p21 pathway and suppression of the cdc25 family phosphatase activity are initiated, which results in reduced cdk activity and cell cycle arrest [38,42,55]. Indeed, in addition to cyclin binding, cdk activity is also regulated by a critical phosphorylation/dephosphorylation equilibrium and counteracted by cell cycle inhibitory proteins, called the cdk inhibitors (cdki) [42,51,55] (Fig. 1). Based on their structure and the identity of their cdk targets, two families of cdki have been described, namely the Ink4 family and the Cip/Kip family. The former comprises four distinct proteins, namely p15, p16, p18, and p19, which are specific inhibitors of cdk4/6. The Cip/Kip family proteins, including p21, p27, and p57, bind and inhibit different cdk/cyclin complexes [42,55].

Hepatocyte senescence

Following partial hepatectomy (PH), the remaining hepatocyte population needs to divide on average 1.6 times before the normal liver mass is restored and the regeneration is put back on hold [26,31,56]. It has been suggested that TGFβ and activin A, known inhibitors of hepatocyte proliferation, as well as extracellular matrix-driven signals, underlie the termination of hepatocyte growth when the liver regeneration is completed [57–59]. During chronic liver injury, human hepatocytes are repeatedly stimulated to proliferate due to iterative waves of liver destruction and regeneration [60]. This *in vivo* proliferation capacity was further highlighted by the efficient repopulation of *Fah*$^{-/-}$/*Rag2*$^{-/-}$/*Il2rg*$^{-/-}$ mice with human adult hepatocytes for at least four sequential rounds [61]. However, human hepatocytes cannot proliferate indefinitely. Liver cirrhosis is accompanied by a significant rate of hepatocellular senescence and characterized by considerable short hepatocyte telomeres [60]. In humans, telomerase activity of most cell types is repressed early during development. Consequently, telomere DNA in proliferating somatic cells undergoes progressive attrition. Once a critical minimal length is reached, cellular growth is arrested irreversibly, a process known as replicative senescence, which was first described by Hayflick and Moorhead nearly 50 years ago [2,62–64]. One way to overcome telomere-dependent senescence is by reactivating the telomerase activity with exogenous hTERT [65–67]. In contrast to humans, rodents display substantial telomerase activity in several somatic tissues, including the liver [62,68–71]. Their telomerase activity increases 24 h after PH and is enhanced by the preoperative treatment with EGF and HGF, but repressed by MAPK kinase inhibitors [72]. In primary rodent hepatocyte cultures, upregulation of telomerase activity was only notable or further enhanced after addition of growth factors to the culture medium [70,72]. The high regeneration capacity, characteristic of rodent livers, may be linked to this strong telomerase activity [71]. In this regard, serially transplanted adult mouse hepatocytes have been demonstrated to divide as many as 69 times [31,73].

However, *in vitro*, both human and rat adult hepatocytes do not possess spontaneous cell growth and their proliferation capacity remains usually quite limited even when cultured under growth promoting conditions [31,36,68,74–76]. The *in vitro* premature growth arrest, observed in primary hepatocyte cultures could be related to a telomere-independent senescence mechanism, which remains to be fully elucidated, but is suggested to involve tumour suppressor proteins and cdkis [63,77]. Indeed,

Table 1. Overview of the functionality and immortalization strategy of *in vitro* established human and rodent hepatic cell lines.

Human hepatic cell lines			
Cell line	Immortalization strategy	Functionality	[Ref.]

Fa2N-4	Adult hepatocytes
	Transfection
	SV40 Tag
		• Possessed, in comparison with cryopreserved human hepatocytes:
		  • Significantly lower basal mRNA expression levels of the nuclear receptor *CAR* and several drug metabolizing enzymes and transporters, namely *CYP1A2/2D6/2E1/1A*1, *UGT1A1/1A6/2B15/2B4*, sulfotransferase, *SLC10A1, SLC22A1, SLCO1B1/1B3, ABCC2 and ABCB11*
		  • Markedly higher *ABCB1* mRNA levels
		  • Similar basal expression of *ABCG2, PXR and AhR*
		  • Apparently higher expression of most transcription factors and coactivators/ corepressors that have been associated with PXR and CAR mediated enzyme induction
		• Were incapable of metabolizing compounds due to low basal levels of drug-metabolizing enzymes
		• Exhibited, at early passage, inducible *CYP1A2/2C9/3A4, UGT1A* and *ABCB1* mRNA levels as well as CYP1A2/2C9/3A4 activities and could distinguish inducers from non-inducers. At higher passages, the cells lost the ability to induce
		• Were not tumorigenic
			[22, 86, 176]

FH-TERT	Fetal hepatocytes Retroviral vector hTERT	• Expressed *CYP450* mRNA and maintained, in contrast to passaged fetal hepatocytes, liver-enriched differentiation markers, especially *C/EBPα* and *HNF4* as well as elevated levels of *HGFR* • Possessed glycogen storage and G6P activity, in a pattern similar to primary fetal hepatocytes • Produced urea and retained level of ALB synthesis equivalent to HepG2 cells • Displayed no *in vitro* anchorage-independent growth or *in vivo* tumor formation but acquired cytogenetic aberrations (e.g., trisomy 7) in long term cultures • 4 weeks after intrahepatic transplantation in immunodeficient mice, FH-hTERT engrafted, survived and expressed *ALB, A1AT,* and *TF* mRNA levels comparable with primary human hepatocytes. These experiments illustrated the ability of FH-hTERT to differentiate into mature hepatocytes and to display significant hepatocellular gene expression ► Culture conditions used in these studies were designed at supporting cell proliferation, and conditions have not been optimized for inducing differentiated hepatocellular functions	[2, 177]
Hc3716-hTERT	Fetal hepatocytes Retroviral vector hTERT	• Maintained normal mammalian cell morphology • Exhibited protein expression of ALB, CK8 and CK18, but not AFP. ALB levels were higher than in control, passaged Hc3716 cells • Possessed inducible *CYP3A4/7* mRNA levels • Exhibited wild-type p53 responsiveness • Did not show typical oncogenic phenotype traits ► In this study, it is shown that inappropriate culture conditions induce senescence programming in human cells. Adapting the culture medium allowed the human fetal hepatocytes to extend their lifespan over 80 passage doublings, instead of 10 in normal culture conditions	[108]
HepLi5	Adult hepatocytes Retroviral vector SV40 Tag	• Expressed *HBCF-X, GS, GST, ALB* and *CYP450* mRNA • Retained ALB secretion and urea production, though at low levels compared to primary hepatocytes • Dislayed CYP1A2 activity • Were not tumorigenic • Possessed significantly enhanced cellular functions after large-scale culture in roller bottles	[141]
HepLL	Adult hepatocytes Lipid mediated gene transfer (lipofectamine reagent) SV40 Tag	• Displayed morphologic characteristics of liver parenchymal cells • Expressed *HNF4, HBCF-X, GST-π* and *ALB* mRNA as well as ALB and CYP2E1 protein but no *ASGP* mRNA • Stained positive for human hepatocyte special antigen but negative for AFP • Secreted ALB and urea at levels not significantly different from primary cultured human hepatocytes • Synthesized glycogen • Were not tumorigenic after transplantation into SCID mice • Possessed a good potential of regeneration and active metabolic function in recipient organs	[83]

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Table 1 (*continued*)

Cell line	Immortalization strategy	Functionality	[Ref.]
HepZ	Adult hepatocytes Lipid mediated gene transfer (lipofectamine reagent) Antisense constructions for Rb and p53 + Cotransfection of E2F transcription factors and cyclin D1	When grown in bioreactor, cells were able to secrete ALB and A2M and possessed inducible CYP450 activity ► pRB and p53 antisense constructs under control of *ALB* promoter	[112]
HHE6E7T-1/2	Small hepatocytes Lentiviral and retroviral vectors HPV16 E6/E7 + hTERT	• Displayed epithelial-like morphology • Retained characteristics of differentiated hepatocytes, though functions such as ALB secretion as well as mRNA expression levels of *ALB, HNF4* and *A1AT* decreased gradually as the passages progressed. *CK18* mRNA levels were detected throughout the culture period and no *AFP* expression was observed • Were positive for vimentin staining • Showed chromosomal instability after long-term passage • Exhibited no tumorigenic properties after transplantation in SCID mice • Improved survival of acetaminophen-induced ALF mice through possible redifferentiation *in vivo*. It was suggested that once transplanted, cells may support ammonia metabolism and gluconeogenesis, even though cells at latter passages did not possess ureogenesis or gluconeogenesis ► In this study, transduction of hTERT alone could not extend the life span of normal human adult hepatocytes	[20, 153]
HHL(-5/-7/-16)	Adult hepatocytes Retroviral vector HPV16 E6/E7	• Contained markers of hepatocyte and biliary phenotype (CK7/8/18/19) • Expressed CYP450 protein at levels comparable to HuH-7 and HepG2 cells • Produced ALB, though at lower levels than HuH-7 and HepG2 cells • Stained negative for AFP and did not display elevated nuclear expression of p53 protein • Possessed active gap junctions • Responded to IFN-α stimulation by upregulation of major histocompatibility complex I and II • Exhibited, in contrast to the HuH-7 and HepG2 cells, increased capacity to bind recombinant hepatitis C virus-like particles	[19]
IHH-A5	Adult hepatocytes Lipid mediated gene transfer (lipofectin reagent) SV40 Tag	• Were morphologically and functionally more similar to hepatoma cell lines than primary hepatocytes in culture • Secreted different plasma proteins, including ALB, APO-B and fibrinogen at relatively high rates, within the range observed for early primary human hepatocyte cultures. Addition of IL-6 to the culture medium resulted in increased fibrinogen secretion and decreased ALB production, demonstrating a proper acute-phase response • Produced detectable amounts of APO-A1 • Exhibited bile-canalicular structures that, in some cases, accumulated the organic anion glutathione-methylfluorescein. Cell cultures were partly polarized and expressed the efflux transporters, MDR1 and MRP1, on the membranes of apical vacuoles or on the lateral membranes of adjacent, proliferating cells, respectively • Did not maintain active Na^+ -dependent bile salt uptake • Displayed similar lipoprotein metabolism as HepG2 cells	[135]
NeHepLxHT	Neonatal hepatocytes Retroviral vector hTERT	• Displayed characteristic morphology of primary fetal liver cells • Maintained epithelial characteristics as evidenced by immunostaining for epithelial cell markers, the cytokeratins • Possessed gene expression profile similar to human neonatal hepatocytes, with positive expression of *A1AT, CKIT, CLDN3, EPCAM, NCAM* mRNA and no detection of *AFP, ASGPR or CYP3A4*. The very low *ALB* mRNA levels compared to HepG2 cells and the expression of *CK19* in early passages, indicated the progenitor nature of the cells • Retained a normal diploid karyotype	[9]
OUMS-29	Fetal hepatocytes Lipid mediated gene transfer (lipofectin reagent) SV40 Tag	• Displayed epithelial morphology • Maintained gene expression of *ALB, ASGPR, bil-UGT, GS, GST-π, HBCF-X, AhR* and *Arnt* • Secreted ALB, AFP, TF, A1AT and APO-A1 • Possessed inducible *CYP1A1/2* mRNA levels and activity • Displayed chromosomal abnormalities • Protected 90% hepatectomized rats from hyperammonemia and prolonged their survival after intrasplenic transplantation ► Overexpression of HNF4α2 led to development of OUMS-29/H-11 cell line with increased liver-specific gene expression, such as A1AT, apolipoproteins, HBCF-X and HNF1α	[21, 122, 136]

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Table 1 (*continued*)

Cell line	Immortalization strategy	Functionality	[Ref.]
PH5CH	Adult hepatocytes Lipid mediated gene transfer (lipofectin reagent) SV40 Tag	• Displayed epithelial appearance • Expressed human CK and ALB protein • Possessed low colony-forming efficiency • Were not tumorigenic	[137]
THLE-2/-3	Adult hepatocytes Retroviral vector SV40 Tag	• Displayed epithelial morphology • Secreted ALB and expressed CK18, TF, A1AT, A2M, GST-π and very low levels of GGT at early passages. CK19 expression could only be determined at later passages. Cells were uniformly negative for AFP and factor VIII. The appearance of CK19 and decreased ALB secretion at later passages demonstrated that cells undergo dedifferentiation in culture • Retained mRNA expression of phase II enzymes such as *EH*, *catalase*, *GPX*, *SOD* and *GSTs* at levels comparable to human liver, with *GST*-π and *α* mRNA as the dominant form in THLE cells or human liver, respectively • Maintained *NADPH CYP* reductase expression at a lower steady-state mRNA level than in human liver • Were able to metabolize three carcinogens, which suggested the presence and activity of CYP1A2/1A1, CYP2E1 and CYP3A4. However *CYP1A2, CYP2E1, CYP3A4, CYP2A3* and *CYP2D6* mRNA were not detected. The steady-state mRNA levels of *CYP1A1* increased after exposure to Aroclor 1254 or B[α] P. • Displayed chromosomal abnormalities • Possessed no tumorigenic properties ► Besides the use of viral vectors, strontium phosphate transfection was also applied (THLE-1 cells). Immortalization could only be reproducibly established by retroviral transduction	[104]
TPH1	Adult hepatocytes Strontium phosphate precipitation HCV core gene	• Exhibited altered cell morphology resembling low-differentiated epithelial cells • Expressed no *A1AT* or *AFP* mRNA • Secreted ALB • Possessed G6P activity • Reactivated telomerase immediately after senescence • Displayed anchorage-independent growth at latter stages, providing evidence for transformed phenotype	[113]
Conditional immortalization			
16T-3	Adult hepatocytes Retroviral vector hTERT Tamoxifen-mediated self-excision (Cre-LoxP)	Reverted 16T-3 cells: • Showed enhanced mRNA levels of transcriptional factors, *C/EBPα* and *HNF4α* as well as increased mRNA expression of hepatocyte-specific genes, including *ALB, GST*-π, *HBCF-X, bil-UGT, CYP3A4, GS* and *ASGPR* • Possessed increased ALB production and lidocaine metabolism, though at lower levels than normal human hepatocytes • Intraportal transplantation in a pig model of ALF induced by D-galactosamine recovered TBL, AST, NH_3 and PT levels and prolonged survival without tumor formation	[111]
cBAL111	Fetal hepatocytes Lentiviral vector hTERT Transcriptional regulation (Tet-on approach)	• Expressed relatively high mRNA levels of immature markers, *GST*-π and *AFP*, and very low mRNA levels of mature markers, *ALB, A1AT* and *TF*. Transcript levels of *HNF4α* increased after prolonged culturing • Stained positive for GS, ALB, CK18, CK19, vimentin and the progenitor cell marker CD146 but displayed CK18 in a pattern characteristic of dedifferentiated human hepatocytes • Produced urea and ALB, though at lower levels than mature human hepatocytes. • Retained no CYP1A2 & 3A4 activity (no elimination of lidocaine) but were able to eliminate galactose • Displayed no anchorage-independent growth • Possessed the ability to differentiate into functional hepatocytes once transplanted *in vivo*, without the occurrence of tumor formation ► cBAL111 cells resembled cells with progenitor characteristics rather than fully differentiated hepatocytes. However, there was a trend of increased and decreased expression of mature and immature markers, respectively, with culture time	[7]
HepCL	Fetal hepatocytes Retroviral vector SV40 Tag Temperature-based regulation	• Displayed morphological characteristics of liver parenchymal cells • Stained positive for ALB, CK18 and CK19 • Produced amounts of ALB and urea comparable to those of unmodified primary human fetal hepatocytes • Were not tumorigenic after transplantation into SCID mice • Transplanted cells rescued mice after 90% hepatectomy, produced ALB and CK18 and were superior to HepG2 cells regarding metabolic support during ALF	[124]

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Table 1 (*continued*)

Cell line	Immortalization strategy	Functionality	[Ref.]
HepLi-4	Adult hepatocytes Retroviral vector SV40 Tag Tamoxifen-mediated self-excision (Cre-LoxP)	Reverted HepLi-4 cells: • Expressed similar *GS* and somewhat lower *UGT1A1* mRNA levels than adult human liver. *ALB* and *GST*-π mRNA levels were extremely lower or higher, respectively, compared to the human liver. This indicates that HepLi-4 cells are not fully differentiated after reversion • Prolonged the survival of common bile duct ligated mice after intrasplenic transplantation • Were not tumorigenic	[87]
HLTC-7/ -11/-15/-17/ -19	Adult hepatocytes Retroviral vector SV40 Tag Temperature-based regulation	• Grew as islands or sheets of cuboidal cells (HLTC-17) or displayed a more dispersed cuboidal-elongated morphology (HLTC-7/-11/-15/-19) • Secreted fibrinogen at fairly constant rate in all tested cell lines at permissive (33.5°C) and non-permissive (39.5°C) temperature • Exhibited no ALB, AFP, A1AGP or PT secretion in any cell line at both temperatures • Cell lines HLTC-7,-15 and -19, produced A1AT at permissive temperature. However, at non-permissive temperature the secretion of A1AT was upregulated or became detectable in all the cell lines • Cell lines HLTC-17 and -11 possessed no CYP activity at any temperature even after induction and stained positive for ALB, CK18, CK7, CK19 and vimentin, but negative for CK8, with almost identical patterns at both temperatures • HLTC-17 cells showed considerable aneuploidy with chromosomal rearrangements. All HLTC cells contained a derivative chromosome with loss of the short arms of chromosomes 11 and 12 • Displayed no anchorage-independent growth ► The results indicated progressive phenotypic instability and loss of differentiated functions. Conversion to the non-permissive temperature did only allow significant expression of a limited repertoire of differentiated functions by the immortalized human hepatocytes	[103]
IHH10(.3)/12	Adult hepatocytes Lentiviral vector SV40 Tag + hTERT (IHH10) or SV40 Tag + hTERT + Bmi-1 (IHH12) Recombinase- based control (Cre-LoxP)	• Displayed morphology reminiscent of differentiated hepatocytes • Expressed *ALB, A1AT, ASGPR* and *CYP450* mRNA levels • Secreted liver-specific proteins, ALB and fibrinogen, at levels similar to HuH-7 cells but lower than primary hepatocytes. The IHH12 cell line did only produce fibrinogen after de-immortalization, suggesting the acquirement of a higher differentiation status in this setting. However, Cre-recombinase treatment of IHH12 cells did not significantly improve the production of ALB • Possessed inducible CYP1A1/2 activity • Were not tumorigenic and rescued mice from lethal doses of acetaminophen ► Combination of immortalizing genes hTERT & Bmi-1 was insufficient to immortalize non-dividing human hepatocytes	[23]
NKNT-3	Adult hepatocytes Retroviral vector SV40 Tag Recombinase-based control (Cre-loxP)	• Displayed morphological characteristics of liver parenchymal cells and looked more differentiated after reversion • Expressed *bil-UGT*, *GS* and *GST*-π mRNA levels, which increased substantially after reversion. Contradicting results were published regarding expression of *ALB* and *HBCF-X* mRNA levels. One paper demonstrated that *ALB* and *HBCF-X* mRNA were newly introduced in the reverted cells whereas several other papers already reported expression of these genes and *ASGPR* mRNA in non-reverted cells. Nevertheless, although reversion did stimulate differentiation, mRNA levels of *ALB*, *A1AT* and *TF* were maximally 0.1% of primary human hepatocytes • Were not tumorigenic after transplantation into SCID mice and both NKNT-3 and reverted NKNT-3 significantly improved biochemical parameters in transplanted rats, protecting as such 90% hepatectomized rats from hyperammonemia and prolonged their survival ► Additional experiments revealed that introduction of p21 into human immortalized hepatocytes can increase ALB expression and induce a differentiated morphology	[6, 85, 117, 136, 140, 152]
YOCK-13	Adult hepatocytes Retroviral vector hTERT Tamoxifen-mediated self-excision (Cre-LoxP)	• Displayed morphological characteristics of normal human hepatocytes. • Expressed markers of hepatocytic differentiation including ALB, ASGPR, bil-UGT, CYP3A4, GS, GST-π, and HBCF-X • Immortalized and reverted cells possessed no tumorigenic properties in SCID mice • Xenotransplantation in totally pancreatectomized pigs, decreased hyperglycemia and prolonged survival without adverse effects such as portal thrombosis, liver necrosis, pulmonary embolism and tumor development ► The YOCK-13 hepatic cell line is derived from the reversible immortalized human hepatic cell line, TTNT-16-3, by co-expression of modified insulin	[110]

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Table 1 (*continued*)

Rodent hepatic cell lines			
Cell line	Immortalization strategy	Functionality	[Ref.]
AdPX3/4	Rat adult hepatocytes Calcium phosphate precipitation E1A & E1B	• Secreted ALB at early passage, though at lower levels than primary cultured hepatocytes and many CWSV cell lines. Moreover, ALB amounts declined with continued passage in culture. The cells also produced additional plasma proteins, including TF, hemopexin and C3 complement but no AFP • AdPX4 cells were, at least at low passage, not tumorigenic ► Use of *E1A* or *E1B* gene expression alone was insufficient to induce hepatocyte proliferation ► Transformation frequencies were enhanced when growth factors were added to the culture medium	[25]
C3-II-B-2-3 C4-1-B-2 C8-IV P9 SV40RH1	Rat adult hepatocytes Calcium phosphate precipitation SV40 DNA	• Produced ALB (SV40RH1/C3-II-B-2-3/C4-1-B-2/C8-IV) • Possessed bil-UGT activity (SV40RH1/P9) • Were able to resynthesize gluthatione from methionine and cysteine (SV40RH1/P9) • Expressed low levels of phase II enzymes, GGT and GST-π (SV40RH1/P9) • Contained cells expressing vimentin. The SV40RH1 was the only cell line in which vimentin-negative cells were present	[133]
CWSV	Rat adult hepatocytes Calcium phosphate precipitation SV40 DNA	• Were derived from colonies of ALB-producing epithelial cells, which were morphologically more similar to established hepatoma cell lines than cultured primary hepatocytes • At low passage, the cell lines, CWSV1, 2, 14, 16 and 17 expressed high *ALB* mRNA levels and possessed *TF, A1AT* and *PEPCK* mRNA levels which were similar to the liver but higher than to 2 hepatoma cell lines (H4IIEC3 & McA-RH7777). At higher passage *ALB* expression decreased only slightly in the CWSV1, 2 and 17 cell lines but markedly in CWSV14 and 16 cells. *TF* levels were marginally diminished in all cell lines whereas *A1AT* and *PEPCK* were only highly maintained in CWSV1 and 14 cells, respectively. No *AFP* expression could be detected throughout cultivation, except in high passaged CWSV14 cells • Exhibited variable ALB production, ranging from high (CWSV1, 2, 9, 10, 14, 15, 16 and 17) to low (CWSV4 and 5) levels. CWSV8 cells did not produce detectable ALB amounts from passage 11 onwards. ALB-producing cell lines also secreted other plasma proteins, including, TF, hemopexin and C3, but no detectable amounts of AFP • CWSV1, 2, 16 cells were only tumorigenic at higher passages whereas transplanted CWSV 14 and 17 cells already formed tumors at low passages ► Transformation frequencies were enhanced when growth factors were added to the culture medium ► It was shown that culture conditions used for transformation could greatly influence the differentiated hepatic phenotype of transformed cells. For example formation of ALB secreting colonies was greatly increased after addition of DMSO to the culture medium ► Experiments demonstrated that ALB expression was apparently temperature-independent	[105, 134, 178]
RH(1-4/6-10)	Rat adult hepatocytes Electroporation SV40 Tag	• Displayed morphology consistent with that of immature hepatocytes and identical to certain cultured undifferentiated hepatoma cells • Did not express CK19 protein levels • Retained ALB-positive cells, but mRNA and protein expression were weaker than in rat hepatocytes • Showed AFP protein expression in some cell strains (RH8/9/10), but to a lesser extent than HepG2 cells. The functional status of these RH cells was thought to resemble that of 10- to 17-day-old fetal rat hepatocytes ► Establishment of unique method to specifically immortalize ALB-expressing cells. Cultures were cotransfected with puromycin resistance gene under control of *ALB* enhancer/promoter. Only cells derived from hepatocytes were obtained after drug selection	[24]
SVHepB4	Rat adult hepatocytes Wild type simian virus strain LP SV40 Tag	• Displayed a hepatocyte-like morphology • Maintained significant activity and inducibility of phase I and phase II enzymes. • Showed low activity of GGT, a hepatocyte dedifferentiation or transformation marker, whereas the hepatospecific enzyme TAT was expressed at levels similar to those in liver • Possessed no anchorage-independent growth	[179]

(*continued on next page*)

Table 1 (*continued*)

Conditional immortalization			
Cell line	Immortalization strategy	Functionality	[Ref.]

	Rat adult hepatocytes
	Retroviral vector
	SV40 Tag
	Temperature-based regulation
		• Immortalized cells cultured at permissive temperature (33°C) expressed low mRNA and protein levels of ALB, ASGPR and androsterone-UGT but high mRNA and protein levels of GST-Yp, compared to 24 hours primary rat hepatocyte cultures
		• Immortalized cells cultured at non-permissive temperature (37°C):
		  • Displayed morphologic characteristics of differentiated hepatocytes
		  • Expressed increased mRNA and protein levels of ALB, ASGPR, and androsterone-UGT
		  • Possessed markedly decreased GST-Yp expression
		• Transplantation into SCID mice revealed no tumorigenic properties and cells retained hepatocellular morphology and G6P activity
		► Expression of ASGR was found to be temperature-sensitive with higher expression at 37°C compared to 39°C. Consequently after SV40 Tag degradation, cells were maintained at 37°C instead of 39°C
			[118]

	Rat adult hepatocytes Human artificial mini chromosome SV40 Tag Recombinase-based control (FLP/FRT)	• Displayed morphology of primary hepatocytes • Expressed *ALB, G6P, DPP-IV, GGT* mRNA levels similar to normal hepatocytes and *ALB* levels were stable for at least a month • Secreted an amount of ALB equivalent to primary hepatocytes, however once transplanted in hepatectomized nonalbumin rats, immortalized hepatocytes did not generate significantly elevated ALB levels compared with primary hepatocytes. • Were not tumorigenic	[129, 147]
BQ1 BV1 WA1 WB6	Rat adult hepatocytes Retroviral vector SV40 Tag Temperature-based regulation	• Displayed typical epithelial cell morphology • Cultured at non-permissive temperature (37°C) expressed decreased levels of p53 • Although increased at non-permissive temperature, production of ALB and urea was still low compared to primary hepatocytes and reached undetectable levels with passage • Possessed increased telomerase activity following immortalization but no anchorage-independent cell growth was visible • Distribution of chromosomes was adapted during passaging. Significant increase of tetraploid along with passage (WA1/WB6/BQ1). For BV1 cells no significant change of chromosome count with passage, though several common chromosomal aberrations, namely, trisomy, monosomy and unknown marker chromosome were noted only in higher passages ► It was demonstrated that conditionally immortalized hepatocytes become dedifferentiated by *in vitro* passage	[102]

C8-B	Rat adult hepatocytes
	Retroviral vector
	SV40 Tag
	Recombinase-based control (*Cre-LoxP*)
		• Following Cre-recombinase treatment:
		  • Regained morphological characteristic of differentiated hepatocytes
		  • Showed increased mRNA levels of *ALB, HNF4* and *UGT1* and newly detectable *UGT2* and *ASGPR* mRNA
		• Possessed no anchorage-independent colony formation or tumor production.
		► Ad-Cre infection was not 100% efficient and some cells did not undergo recombination
			[84]

H2.35	Mice adult hepatocytes Simian virus 40 SV40 Tag Temperature-based regulation	• Cultured at permissive temperature exhibit extremely low *ALB* mRNA levels • Cultured at non-permissive temperature in serum-free medium and collagen substratum possessed dramatically increased *ALB* mRNA levels	[120]
L2A2	Rat adult hepatocytes Retroviral vector SV40 Tag Temperature-based regulation	• Cultured at non-permissive temperature (37°C/39°C) regained the morphological characteristics of differentiated hepatocytes • Possessed increased ALB production at non-permissive temperature • Integrated normally into line cords and appeared indistinguishable from native liver parenchymal cells when transplanted into normal rat liver • Once engrafted in the spleen of portacaval-shunted rats, displayed normal morphology, secreted bile, expressed *ALB* mRNA and offered protection from hyperammonemia-induced hepatic encephalopathy • Were not tumorigenic	[119, 180]

A1AGP, α1-acid glycoprotein; A1AT, α1-antitrypsin; ABC, ATP binding cassette; AFP, α-fetoprotein; AhR, aryl hydrocarbon receptor; ALB, albumin; ALF, acute liver failure; A2M, α2-macroglobulin; APO, apolipoprotein; Arnt, AhR nuclear translocator; ASGP(R), asialoglycoprotein (receptor); AST, aspartate aminotransferase; Bmi-1, B lymphoma Mo-MLV insertion region 1 homolog; CAR, constitutive androstane receptor; C/EBP, Ccaat-enhancer-binding protein; CD, cluster of differentiation; CK, cytokeratin; CLDN, claudin; CYP, cytochrome P450; DMSO, dimethyl sulphoxide; DPP, dipeptidyl peptidase; EH, epoxide hydrolase; EPCAM, epithelial cell adhesion molecule; GGT, γ-glutamyl transpeptidase; G6P, glucose-6-phosphate; GPX, glutathione peroxidase; GS, glutamine synthetase; GST, glutathione S-transferase; HBCF, human blood coagulation factor; HGFR, hepatocyte growth factor receptor; HNF, hepatocyte nuclear factor; HPV, human papillomavirus; hTERT, human telomerase reverse transcriptase; IL, interleukin; INF, interferon; MDR, multidrug resistance protein; mRNA, messenger ribonucleic acid; MRP, multidrug resistance-associated protein; NADPH, nicotinamide adenine dinucleotide phosphate; NCAM, neural cell adhesion molecule; NH_3, ammonia; PEPCK, phosphoenolpyruvate carboxykinase; PT, prothrombin; PXR, pregnane X receptor; Rb, retinoblastoma; SCID, severe combined immunodeficiency; SLC, solute carrier; SOD, superoxide dismutase; SV40 Tag, simian virus 40 large T antigen; TAT, tyrosine aminotransferase; TBL, total bilirubin; TF, transferrin; UGT, uridine diphosphate-glucuronosyltransferase.

Table 2. Overview of the available immortalization strategies.

Immortalizing genes			
	Rodent adult hepatocytes	Human adult hepatocytes	Human fetal hepatocytes
Viral oncogenes	• Allow the cells to overcome the proposed *in vitro* telomere-independent growth arrest • Immortalization of cells	• Allow the cells to overcome the proposed *in vitro* telomere-independent growth arrest • Expansion of *in vitro* lifespan	• Expansion of *in vitro* lifespan
hTERT	n.a	• Contradicting results available • Does not allow the cells to overcome the proposed *in vitro* telomere-independent growth arrest	• Immortalization of cells
Viral oncogenes + hTERT	n.a	• Immortalization of cells	• Immortalization of cells • Use of viral oncogenes may potentially help cells to overcome premature growth arrest which can occur when cultivated under inappropriate culture conditions [77, 108]

Conditional immortalization		
	Advantage	Disadvantage
Temperature-based regulation	• Cell growth can easily be manipulated by temperature shift • SV40 Tag not active at physiological temperature	• The method is restricted to the temperature-sensitive SV40 Tag mutant • The immortalization gene is not removed from genome leading to potential risk for tumorigenesis when used *in vivo* • Temperature shift can induce changes in cellular properties and complicate interpretation of study outcome
Recombinase-based regulation	• Excision of immortalizing gene upon recombinase expression offers more possibilities for *in vivo* applications	• Associated with an irreversible growth arrest • Proper reversion depends on efficient transfer of recombinase gene • Risk for chromosomal rearrangement by recombinase activity
+ negative selection marker (HSV-TK)	Cells that underwent improper recombination can efficiently be eliminated by ganciclovir exposure	
+ tamoxifen-mediated self-excision	Elevates the need of a secondary virus-mediated transfer of the recombinase gene	
Transcriptional regulation	• Allows switching between the proliferating and growth arrest state • Expression of immortalizing gene can be controlled *in vivo*	• Possibility of leaky transgene expression

Gene transfer	
Non-viral	
Calcium phosphate precipitation	High hepatocyte toxicity Low gene transfer efficiencies
Strontium phosphate transfection	Low hepatocyte toxicity Low gene transfer efficiencies
Electroporation	High hepatocyte toxicity Low gene transfer efficiencies
Lipid-mediated gene transfer	When optimized, higher gene transfer efficiencies can be obtained compared to the other non-viral methods Use of hepatocyte-specific ligands as transfection vehicle can lead to more hepatocyte-specific transfections
Viral	
Retroviral	Not able to transduce non-dividing cells
Lentiviral	Transduce both dividing and non-dividing cells Transduction without affecting the differentiated phenotype Improvement of lentiviral transduction efficiency by addition of growth factors and vitamin E to the culture medium
Human artificial chromosomes	Lower transfer efficiency than viral vectors Mitotically stable episomal maintenance Allows incorporation of large genes under control of their regulatory elements

HSV-TK, herpes simplex virus thymidine kinase; hTERT, human telomerase reverse transcriptase; n.a., not applicable; SV40 Tag, simian virus 40 large T antigen.

several studies support the contribution of cdki p21 and/or p16 to the inhibition of DNA synthesis in primary hepatocyte cultures [78–82]. In this respect, it was demonstrated that the second cell cycle G1 block caused by chronic MAPK pathway activation in mitogen stimulated primary hepatocyte cultures is partly related to p21 induction. Of note, transient MAPK pathway inhibition

allows the establishment of multiple replication rounds in these hepatocyte cultures [79].

Hepatocyte immortalization strategies

Immortalized hepatocytes are defined as a population of indefinitely dividing parenchymal cells that retain critical liver functions [68]. Since mature hepatocytes normally possess only limited growth potential when stimulated *in vitro*, immortalization strategies have been developed based mainly on the transduction or transfection of hepatocytes with well-known immortalizing genes. The most frequently used immortalization methods are (i) overexpression of viral oncogenes, (ii) forced expression of hTERT, or (iii) a combination of both [9,68]. Moreover, some other immortalization genes as well as conditional approaches for hepatocyte immortalization have been described (Fig. 2, Tables 1 and 2).

Immortalization genes

Viral oncogenes

Viral oncogenes include the adenoviral E1A/E1B genes, the simian virus 40 large T antigen (SV40 Tag) and the human papillomavirus 16 (HPV16) E6/E7 genes [68]. All of them have been used to establish hepatocyte-derived cell lines, such as C8-B, HepLL, HHL, AdPX3/4, Fa2N4, HepLi-4, and NKNT-3, suggesting that overexpression of viral oncogenes may be sufficient to overcome the premature *in vitro* growth arrest of cultured hepatocytes [19,25,83–87]. These viral oncogenes typically interfere with cell cycling by inhibiting the p16/pRB and p53 pathways [39,68]. Hepatic cell lines have also been developed from livers of transgenic rodents overexpressing the SV40 Tag [88–90].

While the use of viral oncogenes, such as SV40 Tag, has been shown to be sufficient to immortalize rodent cells, overexpression of these oncogenes in human cells most likely only extends lifespan. Immortalization *per se* requires telomerase reactivation either through mutations or by the use of a second immortalizing gene, hTERT [2,10,20,23,68,91,92]. Furthermore, the use of a combined strategy involving a viral oncogene and hTERT, has also been reported to produce more genetically stable cells [11,67,68,93,94]. Indeed single use of viral oncogenes has often been demonstrated to induce chromosomal abnormalities [95–101]. Even though karyotype analysis of newly produced hepatic cell lines has not routinely been performed, chromosomal abnormalities have been described in different cell lines even with combined immortalization [20,21,102–104]. It is important to mention, however, that activation of an additional oncogene, such as Ras is usually needed to observe tumourigenicity [84,105–107].

Human telomerase reverse transcriptase

The single use of hTERT for immortalization has been suggested to avoid some of the genetic and phenotypic instabilities related to the use of oncogenes but is limited to a number of human cell types, including fetal and neonatal hepatocytes [2,6,7,9,10,108,109]. Unlike adult hepatocytes, these immature cells can still proliferate *in vitro* and hence do not need cell cycle stimulation for immortalization [2,6,7,9,49,109]. However, fetal and neonatal human hepatocytes do not possess indefinite growth potential because inactivation of telomerase causes replicative senescence. Consequently, they require overexpression of hTERT to become immortalized [2,7,9,109].

Contradicting results have been reported when only hTERT was used for immortalization of human adult hepatocytes [20,110,111]. As telomerase activity probably does not allow adult hepatocytes to overcome the proposed telomere-independent growth arrest, overexpression of hTERT may be insufficient to drive adult hepatocytes through the cell cycle [5,7,66,68].

Miscellaneous immortalization genes

Specific combinations of immortalization genes, such as SV40 Tag with hTERT and B lymphoma Moloney Murine Leukemia virus (Mo-MLV) insertion region 1 homolog (*Bmi-1*), have been used to immortalize mature human hepatocytes. Bmi-1, like the viral oncogene HPV16E7, is involved in the inactivation of the p16/pRB pathway. On the other hand, simultaneous transduction with Bmi-1 and hTERT appears insufficient to immortalize the non-dividing hepatocytes [23]. Likewise, a combined HPV16E7/hTERT approach did not promote unlimited growth of human adult hepatocytes [20]. A particular cell line has been produced by co-transfection of human adult hepatocytes with p53 and pRB antisense constructs and plasmids that include E2F and cyclin D1 genes [112]. Furthermore, it seems that the hepatitis C core protein can also specifically immortalize mature human hepatocytes [10,113,114]. This core protein is able to induce *c*-Myc and cyclin D1 expression in primary human hepatocytes *via* activation of the signal transducer and activator of transcription-3 pathway [115].

In general, most of the generated hepatocyte-derived cell lines are not tumorigenic, but display reduced or only limited liver-specific functionality [7,20,102]. Taking into account that proliferation and differentiation are mutually exclusive *in vitro*, it has been shown that overexpression of the cdki p21 and the use of conditional immortalization strategies can stimulate to some extent differentiation of the cells [6,23,84,85,102,111,116–120]. Other anti-dedifferentiation strategies developed to counteract the loss of functionality in primary hepatocyte cultures, including co-culture systems and overexpression of liver-specific genes have also proven useful [121,122].

Conditional immortalization strategies

Conditional immortalization enables the development of growth-controlled cell lines. At least three strategies have been reported to conditionally immortalize hepatocytes, namely (i) temperature-based regulation, (ii) recombinase-based regulation and (iii) transcriptional regulation. All these methods rely on the observation that hepatocyte proliferation only takes place when immortalizing genes are expressed [10] (Fig. 2, Tables 1 and 2).

Temperature-based regulation

This method uses a temperature-sensitive SV40 Tag mutant. The immortalizing gene is expressed and active only at the permissive temperature (33 °C), leading to the proliferation of hepatocytes. At higher temperatures (37–39 °C), the immortalization gene is inactivated and cell cycle progression is no longer stimulated [10]. As no other temperature-labile immortalizing genes have yet been identified, this method is confined to SV40 Tag [10]. Moreover, the use of this strategy is not accompanied by the excision of the immortalization gene from the genome and thus could present a potential risk of tumorigenesis

[84,106,123]. Nevertheless, some conditionally immortalized hepatic cell lines are based on this principle, and these cell lines can be transplanted efficiently in rat models of acute liver failure and chronic hepatic encephalopathy, usually without occurrence of tumourigenicity [88,90,102,118–120,124,125]. However, concerns related to tumourigenicity form an important restriction to the clinical appreciation of immortalized human hepatocytes [20]. Importantly, the temperature shift associated with this methodology might induce changes in cellular properties, which can complicate the interpretation of the study outcome. A more sophisticated system, based on recombinase regulation, is thought to offer a solution for these issues [10,88,118,126,127].

Recombinase-based control

The site-specific recombinase strategy uses recombinase expression to excise chromosomal DNA segments flanked by two recombination sequences and thereby irreversibly reverts immortalization [10,128]. Numerous site-specific recombination systems, including the Cre-loxP and the FLP-FRT system, have been used to establish reversible immortalization. These systems have different efficiencies, whereby the Cre-loxP system stands out [123,128]. In this system, immortalization genes are flanked by two identical DNA sequences, called LoxP sites. The excision of these genes is regulated by Cre recombinase [68,123]. Proper reversion thus depends on the efficient transfer of the recombinase gene [10]. More recently, a new method based on tamoxifen-mediated self-excision has been established, rendering secondary virus-mediated transfer of the recombinase gene superfluous [87,110,111]. Furthermore, the suicide gene herpes simplex virus thymidine kinase (HSV-TK) has been introduced in the recombination construct as negative selection marker. Using this strategy, cells that still express the immortalization gene and HSV-TK gene, due to improper recombination, can be eliminated by exposure to ganciclovir [23,123]. Reversible immortalization of numerous hepatocyte-derived cell lines, including C8-B, NKNT-3, IHH, and 16T-3 depends on this recombinase-based control approach [23,84,85,110,111,129,130].

Transcriptional regulation

In this method, immortalization reversibility is obtained by transcriptional control of immortalization gene expression and not by recombinase activity. In this way, the risk of chromosomal rearrangement is avoided and repeated cycles of hepatocyte proliferation and growth arrest are allowed [10,126,127]. Transcription of immortalizing genes can be controlled by using an artificial promoter/transactivator system, such as the well-known tetracycline system [10]. Two approaches are currently available, the tet-off and the tet-on system, which are composed of a tetracycline-regulated promoter and a tetracycline transactivator (tTA) or reverse tetracycline transactivator (rtTA), respectively. When doxycycline is added to the cell culture medium, it binds to the transactivator. In the tet-on systems, bound rtTA interacts with the tetracycline-regulated promoter and induces the expression of the regulated gene. When using the tet-off method, immortalization genes are expressed in the absence of doxycycline, since only unbound tTA can interact with the gene promoter [126,131]. The tet-on approach has been successfully used to produce a fetal liver cell line [7]. A drawback of this method, however, is the possible leaky transgene expression caused by undesired rtTA-tetracycline promoter binding in the absence of doxycycline [126,131]. A tighter regulation of the transgene expression can be obtained by combining the rtTA system with a tetracycline-controlled transcriptional silencer [131].

Gene transfer

An effective gene transfer method is of utmost importance for immortalizing hepatocytes [91]. Different non-viral and viral methods have been used to generate immortalized hepatocyte-derived cell lines, namely plasmid transfection, viral transduction and the use of human artificial chromosomes (Table 2).

Plasmid transfection

Various approaches are available for transfecting plasmids into primary hepatocytes [91,132]. Due to immortalization, stably transfected cells are selected, allowing simple transfection procedures to be used [132]. Examples of common transfection methods that have been used to immortalize hepatocytes include calcium phosphate precipitation and electroporation [24,25,133,134]. However, both approaches typically display low gene transfer efficiencies and high hepatocyte toxicity [91,132]. Replacement of calcium by strontium eliminates toxicity but the gene transfer efficiency remains low [91]. Other researchers explored liposomes as gene carriers for hepatocyte immortalization [21,83,112,135–137]. When properly optimized, lipid-mediated gene transfer can achieve high gene transfer efficiencies compared to other transfection approaches [91]. Furthermore, using hepatocyte-specific ligands, more hepatocyte-specific transfections can be achieved [132].

Viral transduction

Transduction with viral particles covers a widely used methodology for gene transfer. Among the available viral vectors, retroviral and lentiviral vectors induce stable integration of the immortalization gene and thus generate sustained transgene expression in the progeny [132,138]. Furthermore, these vectors do not provoke harmful immune responses and allow integration of large genes [139]. Retroviral vectors, such as the Mo-MLV-derived vectors, have been frequently used to establish human and rodent hepatic cell lines [2,9,19,84,87,102–104,108,110,111,118,119,124,140,141]. A major flaw in this system is its inability to transduce non-dividing cells, which makes it unsuitable for non-proliferating cells, including hepatocytes [139,142]. Even when growth factors are added to the cell culture medium to induce hepatocyte mitosis, the efficiency of transduction often remains limited [132,139,142,143]. Lentiviral vectors derived from the human immunodeficiency virus (HIV) can tackle these issues and transduce both dividing and non-dividing cells by using virus at a relatively high titer [139,142–144]. Moreover, lentiviral vectors can provide high transduction efficiencies without affecting the differentiated hepatic phenotype [139,143,145]. Although lentiviral vectors lack hepatocyte specificity, the use of hepatocyte specific promoters can restrict the expression of lentiviral genes to the parenchymal liver cells [144]. Several studies have demonstrated appropriate gene transfer for immortalization of human adult and fetal hepatocytes [7,20,23]. Rodent hepatocytes, especially murine hepatocytes are considerably resistant to HIV vector-mediated transduction. This resistance has been related to a block in the immediate-early phase of infection [142]. In addition to the use of higher viral titers, cell culture medium supplied with growth factors, namely EGF and to a lesser extent HGF, was found to improve lentiviral transduction efficacy of primary mouse

hepatocytes [142,146]. Similarly, when transducing human adult and fetal hepatocytes, the use of growth factors markedly upregulated the expression of lentiviral genes. Consequently, this transduction approach offers the possibility to reduce the viral load, which as such lowers cost and reduces cellular toxicity [144]. Also the antioxidant, vitamin E proved to significantly enhance lentiviral transduction rates of human and rat adult hepatocytes [142].

Human artificial chromosomes

The generation of a particular rat hepatic cell line was made possible by a more recent gene transfer method, namely through generation of a human artificial chromosome (HAC) [129,147]. Although this method generally has lower transfer efficiency than the use of viral vectors, the HACs possess many properties of the ideal gene delivery vector. These include mitotically stable episomal maintenance and incorporation of large genes under control of their regulatory elements, allowing a correct, physiologically regulated transgene expression. Furthermore, due to their episomal nature, integration-related complications, such as oncogenesis, should be avoided [138]. Immortalization of human fibroblasts using HAC-mediated episomal expression of hTERT has also been described, potentially offering new perspectives for hepatocyte immortalization [148].

Application of immortalized hepatic cell lines

It has repeatedly been postulated that immortalized hepatic cell lines, which could offer an unlimited supply of well-characterized, pathogen-free cells, may represent an attractive alternative for primary hepatocytes in several clinical applications as well as fundamental and applied research [106,147,149]. So far, multiple studies based on immortalized hepatocytes have already been performed.

Clinical application

Hepatocyte transplantation

The use of different animal models of hepatic impairment made it possible to demonstrate the therapeutic efficiency of transplanted cell lines. In this regard, it was shown that transplantation of conditional immortalized rat hepatocytes could protect portacaval-shunted rats from hyperammonemia-induced hepatic encephalopathy [119,149], improve survival of rats with acute liver failure (ALF) [125], adjust for bilirubin conjugation defect in Gunn rats [150,151], and correct the global hepatic abnormalities associated with end-stage liver failure in cirrhotic animals [149]. Likewise, several human adult and fetal hepatic cell lines, including HHE6E7T1, NKNT-3, IHH, HepCL, 16T-3, and OUMS-29 were confirmed to promote survival in a pig [111], rat [152] or mice [23,124,153] model of ALF. Furthermore, YOCK-13, an insulin-producing human hepatic cell line was reported to control diabetes when transplanted into totally pancreatectomized diabetic pigs [110].

Bioartificial liver systems

For large-scale applications that rely on *in vitro* hepatic functionality, such as BAL systems, the development of a hepatic cell line that combines both *in vitro* hepatic function and proliferation capacity would be of great value.

Two human fetal hepatic cell lines, namely HepLi-4 and cBAL111, have already been evaluated as a potential cell source for BAL systems [87,154]. However, it was revealed that both cell lines possessed insufficient hepatic functionality to be applicable for *in vitro* applications. The need for *in vitro* culture conditions that mimic the *in vivo* situation and promote hepatocyte differentiation *in vitro* was clearly emphasized [7,87,154]. This was further supported by experiments, which showed that cBAL111 cells are able to partly differentiate into functional hepatocytes once transplanted *in vivo* [7].

Different human adult hepatic cell lines have also been proposed as possible candidates for BAL application, but as for the modified fetal hepatic cell line, OUMS-29/H-11, data on efficacy in animal models of severe liver failure are currently lacking [112,141,155–158]. However, the production of ammonia [155] or possible inability to eliminate ammonia [141] are undesirable features for a BAL system [158].

Another modified adult hepatic cell line, composed of TTNT cells overexpressing IL-1 Ra, has already been tested and was not able to improve survival of an ALF rat model [158].

Fundamental and applied research

Nowadays, human and rodent hepatic cell lines, such as CWSV [159,160], H2.35 [161,162], NeHepLxHT [163], OUMS-29 [164], and THLE [165,166] are still being used for fundamental research. In this regard, a lot of investigations related to hepatotropic viruses have been performed on TPH1 cells [167,168]. Furthermore, a murine model of HBV viremia, based on immortalized human hepatocytes transfected with hepatitis B virus DNA, has been created and offers the opportunity for *in vivo* HBV studies [169]. Several hepatic cell lines have also proven useful as *in vitro* tools for screening and safety testing of drug candidates. For instance, Hc3716-hTERT cells represented the first model for predicting the side-effects of telomere-targeting drugs in normal cells and it was suggested that the Fa2N4 cell line could be used for routine screening during discovery for pregnane X receptor mediated CYP3A4 induction [108,170].

Conclusions and perspectives

In vitro expansion of human hepatocytes has gained considerable attention, as it might serve many clinical applications and fundamental research purposes. Prominent examples include the establishment of a bio-artificial human liver device that can be used to bridge the time until liver transplantation is possible and the creation of a liver-based *in vitro* tool for screening and safety testing of drug candidates. As freshly isolated and cultured mature hepatocytes inherently have very poor growth potential, efforts have focused on strategies to immortalize primary hepatocytes while maintaining their liver-specific functions. The currently available methods include transduction or transfection with prototypical immortalization genes and conditional immortalization by temperature-based regulation, recombinase-based control and transcriptional regulation. Although hepatocyte immortalization has been explored for years, it is still in its infancy since no cell lines with high *in vivo*-like hepatic functionality are yet available. As already postulated more attention should be paid to culture systems that support differentiation of the immortalized hepatocytes [6,7,87]. The past decade

witnessed the introduction of novel strategies for cell immortalization, based on the use of cell cycle regulators to surmount the p16-regulated premature growth arrest, observed in several epithelial cells [171,172]. Similarly, human myogenic cells immortalized by combined overexpression of hTERT, cyclin D1 and a mutant cdk4 isoform were able to overcome a p16-regulated precocious growth arrest without loss of their differentiation potential [173]. Although direct sequestration of p16 could not induce hepatocyte proliferation, it is worthwhile to examine the blocking of p16 control and pRB activity by overexpression of cell cycle regulators [20,23]. A prerequisite to develop novel hepatocyte immortalization strategies is further fundamental research on the regulation of liver cell growth, especially *in vitro*. Such efforts should be strongly encouraged as they could lead to the generation of a robust hepatocyte-derived cell line with sustained liver-specific functionality resembling the *in vivo* situation. It can be anticipated that such a system will not only trigger a lot of interest among clinicians but also in the area of *in vitro* pharmacotoxicology.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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