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Immortalized human hepatic cell lines for *in vitro* testing and research purposes

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Summary

The ubiquitous shortage of primary human hepatocytes has urged the scientific community to search for alternative cell sources, such as immortalized hepatic cell lines. Over the years, several human hepatic cell lines have been produced, whether or not using a combination of viral oncogenes and human telomerase reverse transcriptase protein. Conditional approaches for hepatocyte immortalization have also been established and allow generation of growth-controlled cell lines. A variety of immortalized human hepatocytes have already proven useful as tools for liver-based *in vitro* testing and fundamental research purposes. The present chapter describes currently applied immortalization strategies and provides an overview of the actually available immortalized human hepatic cell lines and their *in vitro* applications.

Keywords

cell line; liver; immortalization; human

1. Introduction

At present, primary human hepatocytes represent an important tool for research purposes, in particular in the field of *in vitro* pharmaco-toxicology (1). However, their use is largely impeded by inadequate supply, high cost, high variability and limited *in vitro* proliferation capacity. Alternative cell sources include hepatic cell lines and stem-cell derived hepatocytes (2, 3, 4). Several hepatic cell lines are nowadays available either directly derived from liver tumor tissue or generated from primary hepatocytes *in vitro* (5, 6). Although several hepatoma-derived cell lines, such as HepaRG cells, preserve important liver-specific functions, most of them do not exhibit sufficient *in vivo*-like functionality to be of pharmaco-toxicological relevance (7-12). Immortalized hepatocytes are usually derived from healthy primary hepatocytes by the use of a defined immortalization strategy. Several fetal and adult hepatic cell lines have already been established, whether or not using a combination of viral oncogenes and the human telomerase reverse transcriptase (hTERT) protein (13-19). In this chapter, a number of immortalization strategies are discussed and a state-of-the-art overview of the currently available immortalized human hepatic cell lines and their *in vitro* applications is provided.

2. Hepatocyte immortalization strategies

The most common methods for immortalization of primary hepatocytes are (i) overexpression of viral oncogenes, (ii) forced expression of hTERT or (iii) a combination of both (Table 1) (14, 20). A number of additional immortalization genes as well as conditional approaches for hepatocyte immortalization have also been described (Table 2).

2.1. Immortalization genes

2.1.1. Viral oncogenes—Several human hepatic cell lines have been generated using viral oncogenes, such as the simian virus 40 large T antigen (SV40 Tag) and the human papillomavirus 16 (HPV16) E6/E7 genes, suggesting that overexpression of these viral oncogenes could be sufficient to surmount the premature *in vitro* growth arrest of cultured adult hepatocytes (15, 17, 18, 20-29). Indeed, in contrast to fetal hepatocytes, human adult hepatocytes do not undergo spontaneous cell growth *in vitro* and possess very limited proliferation capacity, even when cultivated in growth-promoting culture systems (6, 20, 30, 31). It has been proposed that this *in vitro* precocious growth arrest could be the result of a telomere-independent senescence mechanism, which possibly involves tumor suppressor proteins and cyclin-dependent kinase inhibitors (32). Viral oncogenes typically promote cell cycling by inhibiting the p16/retinoblastoma protein (pRB) and p53 pathways (20, 33). However, while the use of viral oncogenes, such as SV40 Tag, is sufficient to produce immortalized rodent cells, overexpression of these oncogenes probably only extends lifespan in human cells. Human hepatocytes, like other somatic cells, do not possess telomerase activity and are subject to replicative senescence (20, 32, 34, 35). Consequently, immortalization *per se* requires telomerase reactivation either through mutations or by involving a second immortalizing gene. In particular, hTERT has been used for this purpose (16, 19, 20, 34, 36-38).

2.1.2. Human telomerase reverse transcriptase—The single use of hTERT for immortalization is limited to a number of human cell types, including fetal and neonatal hepatocytes (6, 13, 14, 34, 36, 39, 40). These immature cells are still able to proliferate *in vitro* and hence do not need cell cycle stimulation for immortalization (6, 13, 14, 34, 40, 41). However, fetal and neonatal human hepatocytes do not display indefinite growth potential due to telomere-dependent senescence. As a result, overexpression of hTERT is needed to immortalize these hepatocytes (13, 14, 34, 40). Contradicting results have been obtained when merely hTERT is used to immortalize mature hepatocytes (16, 42, 43). Since telomerase activity most likely does not allow adult hepatocytes to overcome the suggested telomere-independent growth arrest, overexpression of hTERT may not be adequate to stimulate the progression of adult hepatocytes through the cell cycle (5, 13, 20, 44).

2.1.3. Miscellaneous immortalization genes—The hepatitis C core protein has been described as a specific immortalization agent for mature human hepatocytes (36, 45, 46). A particular cell line was developed by co-transfecting human adult hepatocytes with p53 and pRB antisense constructs and plasmids that include E2F and cyclin D1 genes (47). Furthermore, 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 also proven useful for the immortalization of mature human hepatocytes. Bmi-1 has a similar function as the HPV16E7 oncogene and its expression inactivates the p16/pRB pathway. However, the simultaneous transduction with Bmi-1 and hTERT, like for the combined HPV16E7/hTERT approach, appears to be insufficient to immortalize non-proliferating adult hepatocytes (16, 19).

2.2. Gene transfer

Appropriate gene transfer is of utmost importance for hepatocyte immortalization (37). In this regard, both viral and non-viral transfer methods have been used to generate immortalized hepatocyte-derived cell lines.

2.2.1. Plasmid transfection—Since the immortalization process will select cells that stably express the immortalization genes, simple transfection methods can be used (48). At present, various approaches are available for transfecting plasmids into primary hepatocytes (37, 48). The strontium phosphate precipitation method has been used to immortalize human hepatocytes (45). Although this method is cheap and allows robust transfection of primary hepatocytes with low toxicity, it is generally accompanied by limited gene transfer efficiency (37). Liposomes have also been explored as gene carriers for hepatocyte immortalization (17, 22, 23, 25, 47, 49). When properly optimized, lipid-mediated gene transfer strategies can achieve high gene transfer efficiencies compared to the previously mentioned phosphate-precipitation-based transfection approach (37). Furthermore, combination with hepatocyte-specific ligands allows more hepatocyte-specific transfections (48).

2.2.2. Viral transduction—Transduction with viral vectors is a frequently used strategy for gene transfer. Among the available viral vectors, retroviral and lentiviral vectors enable stable integration of the immortalization gene and thus ensure persistent transgene expression in the progeny (48, 50). Furthermore, these vectors do not induce harmful immune responses and are able to integrate large genes (51). Retroviral vectors have often been used to produce human hepatic cell lines (14-16, 21, 24, 26-28, 34, 39, 42, 43, 52). An important drawback of these vectors, however, is their inability to transduce non-dividing cells, which hampers their use for non-proliferating cells, including hepatocytes (51, 53). Although transduction efficiencies generally remain limited even when growth factors are used, it has been reported that the addition of hepatocyte growth factor to the cell culture medium increases the transduction efficiency in primary human hepatocyte cultures (48, 51, 53-55). Lentiviral vectors derived from the human immunodeficiency virus can overcome these flaws and effectively transduce both dividing and non-dividing cells when applied at a relatively high titer (51, 53, 54, 56). Several studies are based upon lentiviral gene transfer for immortalization of human adult and fetal hepatocytes (13, 16, 19). It has been demonstrated that the lentiviral transduction procedure as such does not interfere with the differentiated hepatic phenotype of primary human hepatocytes (57). Moreover, the addition of growth factors to the cell culture medium can markedly enhance the expression of lentiviral genes in both human adult and fetal hepatocytes when low vector titers are used. This transduction approach therefore offers the opportunity to lower the viral load, which in turn reduces cost and cellular toxicity (56). Also, the antioxidant vitamin E is known to promote lentiviral transduction rates of human adult hepatocytes (53).

2.2.3. Human artificial chromosomes—The successful immortalization of rat hepatocytes and human fibroblasts using human artificial chromosomes vectors expressing SV40 Tag or hTERT, respectively, opens new perspectives for human hepatocyte immortalization (58-60). Although the transfer efficiency is generally lower compared to viral vectors, human artificial chromosomes have many characteristics of an ideal gene delivery vector. Indeed, they are able to incorporate complete genomic loci and maintain a mitotically stable episomal expression throughout many cell divisions. Furthermore, due to their episomal nature, integration-related complications, such as oncogenesis, can be avoided (50).

2.3. Hepatic functionality of the immortalized human hepatocytes

In general, most human hepatic cell lines possess reduced or only limited liver-specific functionality. Strategies that are typically used to counteract the loss of functionality in primary hepatocyte cultures, including the establishment of co-culture systems or the overexpression of liver-enriched transcription factors, have also proven beneficial for immortalized human hepatocytes (61, 62). In this context, overexpression of specific cytochrome P450 (CYP) enzymes lies at the basis of the development of the THLE-CYP sublines (63). On the other hand, as differentiation and proliferation are mutually exclusive *in vitro*, overexpression of the cyclin-dependent kinase inhibitor p21 and the use of conditional immortalization strategies were reported to boost to some extent the differentiated phenotype of hepatocytes (19, 29, 43, 64, 65).

2.4. Conditional immortalization strategies

Conditional immortalization supports controlled expansion of cells. At present, 3 different strategies are applied for human hepatocytes, namely (i) temperature-based regulation, (ii) recombinase-based regulation and (iii) transcriptional regulation (36) (Table 2 and 3).

2.4.1. Temperature-based regulation—This method is based upon the application of a temperature-unstable SV40 Tag mutant. At permissive temperature (*i.e.* 33°C), the immortalizing gene is fully active and stimulates hepatocyte proliferation. However, at higher temperatures (*i.e.* 37 to 39°C), the immortalization gene is typically degraded and cell cycle progression is no longer supported (36). Since no other temperature-sensitive immortalizing genes have yet been identified, this method is restricted to the use of SV40 Tag (36). Importantly, the temperature shift related to this methodology might induce variations in cellular properties, which can complicate the interpretation of the study outcome (36, 66, 67). More sophisticated systems based upon recombinase or transcriptional regulation are believed to offer a better solution (68).

2.4.2. Recombinase-based control—The site-specific recombinase strategy results in an irreversible reversion of immortalization, due to permanent removal of immortalization genes (36, 69). The Cre-LoxP site-specific recombination system has often been used to establish reversible immortalization (69, 70). In this method, the immortalization genes are flanked by 2 identical DNA sequences, called LoxP sites, and their excision is regulated by Cre recombinase (20, 70). Hence, proper reversion relies on the effective transfer of the recombinase gene (36). A new method based on tamoxifen-mediated self-excision has been introduced and renders secondary virus-mediated transfer of the recombinase gene superfluous

(26, 42, 43). Additionally, the incorporation of a negative selection marker, such as the suicide gene herpes simplex virus thymidine kinase, allows the removal of cells that underwent improper recombination by exposure to ganciclovir (19, 70). Reversible immortalization of numerous human hepatocyte-derived cell lines, including NKNT-3, IHH and 16T-3 cells, depends on this recombinase-based control approach (19, 42, 43, 71).

2.4.3. Transcriptional regulation—In this method, the reversibility of immortalization does not rely on recombinase activity, but is achieved through transcriptional control of immortalization gene expression. Consequently, repetitive cycles of hepatocyte proliferation and growth arrest are possible and the risk of chromosomal rearrangement is prevented (36, 68, 72). The transcriptional control of immortalizing genes can be accomplished by the use of an artificial promoter/transactivator system, such as the well-known tetracycline system (36). As such, 2 approaches are currently available, namely the tet-off and the tet-on system, which comprise a tetracycline-regulated promoter and a tetracycline transactivator (tTA) or reverse tetracycline transactivator (rtTA), respectively. In the tet-on system, binding of doxycycline to the transactivator will induce the expression of the regulated gene. This is not the case for the tet-off system, since only unbound tTA is able to interact with the gene promoter (72, 73). The tet-on approach has been used for the development of the human fetal liver cell line cBAL111 (13).

3. *In vitro* applications of immortalized human hepatocytes

In recent years, both adult and fetal human hepatic cell lines have been explored for research purposes (Table 1 and 2). Several immortalized human hepatocytes, including PH5CH, TPH1, NKNT-3 and NeHepLxHT cells, have indeed been successfully used as tools in research focused on hepatitis C virus or hepatitis B virus (HBV) (74-81). A murine model of HBV viremia based upon a human hepatocyte-derived cell line transfected with HBV DNA has been described and offers the opportunity for *in vivo* HBV research (82). Human hepatic cell lines have also been applied as cellular models to investigate the processes of hepatocarcinogenesis and steatosis (83-88). Moreover, the HHL cell line proved useful during the development of adeno-associated viral vectors for liver-directed gene therapy (89-91).

Besides their application in fundamental research, different hepatic cell lines are equally addressed as suitable *in vitro* tools for screening and safety testing of drug candidates. In this regard, Hc3716-hTERT immortalized hepatocytes constitute an appropriate *in vitro* model for predicting the side-effects of telomere-targeting drugs (39). Furthermore, Fa2N4 cells may be used as a routine screening system for pregnane X receptor-mediated CYP3A4 induction (92). Similarly, the hepatic THLE cell line and THLE-CYP sublines have been reported as promising models for investigation of CYP-mediated drug metabolism and liver toxicity (63, 93-95). However, NKNT-3 cells appeared to be less suitable than the hepatoma cell line HCC1.2 for the development of improved *in vitro* genotoxicity test systems (96).

4. Conclusion

In vitro expansion of human hepatocytes has gained considerable attention over the years, as it may serve a plethora of fundamental and applied research and screening purposes. Freshly

isolated mature hepatocytes inherently have poor growth potential, a finding that has prompted the search for strategies to immortalize these particular human cells, while maintaining their liver-specific functions. The available methods thus far include transfection or transduction with prototypical immortalization genes and conditional immortalization by temperature-based regulation, recombinase-based control and transcriptional regulation. Although hepatocyte immortalization has been explored for decades, cell lines with *in vivo*-like hepatic functionality are largely lacking. In the upcoming years, more attention should be paid to the search for culture systems that support the differentiation status of immortalized human hepatocytes.

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Table 1
Overview of immortalization strategies, hepatic functionality and in vitro applications of human adult and fetal hepatic cell lines

(A1AT, α 1-antitrypsin; AFP, α -fetoprotein; AhR, aryl hydrocarbon receptor; ALB, albumin; A2M, α 2-macroglobulin; APO, apolipoprotein; Arnt, AhR nuclear translocator; ASGP(R), asialoglycoprotein (receptor); BCRP, breast cancer resistance protein; BSEP, bile salt export pump; CAR, constitutive androstane receptor; C/EBP, Ccaat-enhancer-binding protein; ChREBP, carbohydrate-responsive element-binding protein; CK, cytokeratin; CLDN, claudin; CYP, cytochrome P450; EH, epoxide hydrolase; EMT, epithelial-mesenchymal transition; EPCAM, epithelial cell adhesion molecule; FXR, farnesoid X receptor; GGT, γ -glutamyltransferase; G6P, glucose-6-phosphate; GPX, glutathione peroxidase; GS, glutamine synthetase; GST, glutathione S-transferase; HBCF, human blood coagulation factor; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HGFR, hepatocyte growth factor receptor; HNF, hepatocyte nuclear factor; HPV, human papillomavirus; hTERT, human telomerase reverse transcriptase; IL, interleukin; IFN, interferon; MDR, multidrug resistance protein; mRNA, messenger ribonucleic acid; MRP, multidrug resistance-associated protein; NADPH, nicotinamide adenine dinucleotide phosphate; NCAM, neural cell adhesion molecule; NTCIP, sodium taurocholate cotransporting polypeptide; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; Rb, retinoblastoma; RIPK4, receptor-interacting serine-threonine kinase 4; SOD, superoxide dismutase; SREBP, sterol regulatory element-binding protein; SV40 Tag, simian virus 40 large T antigen; TGF, transforming growth factor; TF, transferrin; (bil-)UGT, (bilirubin-)uridinediphosphate-glucuronosyltransferase).

Adult hepatic cell line			
Cell line	Immortalization strategy	Hepatic functionality of immortalized cells	In vitro applications
Fa2N-4	Transfection	<ul style="list-style-type: none"> - Possess, in comparison with cryopreserved human hepatocytes: <ul style="list-style-type: none"> ■ Significantly lower basal expression level of the nuclear receptor CAR and several drug metabolizing enzymes and transporters, namely CYP1A2/2D6/2E1/1A1, UGT1A1/1A6/2B15/2B4, sulfotransferase, NTCIP, OCT1, OATP1B1/1B3, MRP2 and BSEP. ■ Markedly higher MDR1 mRNA levels. ■ Similar basal expression of BCRP, PXR and AhR. ■ Apparently higher expression of most transcription factors and coactivators/corepressors that have been associated with PXR and CAR mediated enzyme induction. - Are incapable of metabolizing compounds due to low basal levels of drug-metabolizing enzymes. - Exhibit, at early passage, inducible CYP1A2/2C9/3A4, UGT1A and MDR1 mRNA levels as well as CYP1A2/2C9/3A4 activities and could distinguish inducers from non-inducers. At higher passages, the cells lose the ability to induce. 	Routine screening system for PXR-mediated CYP3A4 induction. (18,92,97)
	SV40 Tag		
HepLi5	Retroviral vector	<ul style="list-style-type: none"> - Express HBCF-X, GS, GST, ALB and CYP450 mRNA. 	(21)

Adult hepatic cell line				Reference
Cell line	Immortalization strategy	Hepatic functionality of immortalized cells	In vitro applications	
	SV40 Tag	<ul style="list-style-type: none"> - Retain ALB secretion and urea production, though at low levels compared to primary hepatocytes. - Display CYP1A2 activity. - Possess significantly enhanced cellular functions after large-scale culture in roller bottles. 		
HepLL	Lipid mediated gene transfer (lipofectamine reagent)	<ul style="list-style-type: none"> - Display morphologic characteristics of liver parenchymal cells. - Express HNF4, HBCF-X, GST-II and ALB mRNA as well as ALB and CYP2E1 protein but no ASGP mRNA. 	Testing of new drug carriers for anti-HBV drug delivery.	(22, 98)
	SV40 Tag	<ul style="list-style-type: none"> - Stain positive for human hepatocyte special antigen but negative for AFP. - Secrete ALB and urea at levels not significantly different from primary cultured human hepatocytes. - Synthesize glycogen. 		
HepZ	Lipid mediated gene transfer (lipofectamine reagent)	<ul style="list-style-type: none"> - When grown in bioreactor, cells are able to secrete ALB and A2M and possess inducible CYP450 activity. 		(47)
	Antisense constructions for Rb and p53 under control of ALB promoter + Cotransfection of E2F transcription factors and cyclin D1			
HHIE6/7T-1/2	Small hepatocytes	<ul style="list-style-type: none"> - Display epithelial-like morphology. 		(16, 99)
	Lentiviral and retroviral vectors	<ul style="list-style-type: none"> - Retain characteristics of differentiated hepatocytes, though functions such as ALB secretion as well as mRNA expression levels of ALB, HNF4 and A1AT decrease gradually as the passages progress. CK18 mRNA levels are detected throughout the culture period and no AFP expression is observed. 		
	HPV16 E6/E7 + hTERT	<ul style="list-style-type: none"> - Are positive for vimentin staining. 		
HHL(-5/-7/-16)	Retroviral vector	<ul style="list-style-type: none"> - Contain markers of hepatocyte and biliary phenotype (CK7/8/18/19). 	Used as a cell model to investigate: <ul style="list-style-type: none"> - Ability of CD8+ T-cells to recognise and kill hepatocytes under cytokine stimulation. - Role of sirtuin 4 in energy homeostasis. - Effect of THCV on insulin signalling in insulin-resistant human hepatocytes. 	(15, 89-91, 100-102)
		<ul style="list-style-type: none"> - Express CYP450 protein at levels comparable to Huh-7 and HepG2 cells. 		
	HPV16 E6/E7	<ul style="list-style-type: none"> - Produce ALB, though at lower levels than Huh-7 and HepG2 cells. - Stain negative for AFP and do not display elevated nuclear expression of p53 protein. - Possess active gap junctions. - Respond to INF-α stimulation by upregulation of major histocompatibility complex I and II 		

Adult hepatic cell line					
Cell line	Immortalization strategy	Hepatic functionality of immortalized cells		In vitro applications	Reference
IHH-A5	Lipid mediated gene transfer (lipofectin reagent) SV40 Tag	<ul style="list-style-type: none"> - Exhibit, in contrast to the Huh-7 and HepG2 cells, increased capacity to bind recombinant hepatitis C virus-like particles. - Are morphologically and functionally more similar to hepatoma cell lines than primary hepatocytes in culture. - Secrete 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 results in increased fibrinogen secretion and decreased ALB production, demonstrating a proper acute-phase response. - Produce detectable amounts of APO-A1. - Exhibit bile-canalicular structures that, in some cases, accumulated the organic anion glutathione-methylfluorescein. Cell cultures are partly polarized and express the efflux transporters, MDRI and MRP1, on the membranes of apical vacuoles or on the lateral membranes of adjacent, proliferating cells, respectively. - Do not maintain active Na⁺-dependent bile salt uptake. - Display similar lipoprotein metabolism as HepG2 cells. 	<ul style="list-style-type: none"> - Gene expression and antigen presentation after adeno-associated viral transduction and effect of proteasome inhibition or capsid mutation. 	<p>Used a cell model to investigate:</p> <ul style="list-style-type: none"> - Molecular mechanisms underlying FXR regulation of ChREBP transcriptional activity in human hepatocytes. - Effect of antipsychotic drugs on SREBP transcription factor pathways which control. - Role of c-fos expression on hepatocyte cell motility and cell cycle regulation. - Effect of cadmium on nonmalignant human hepatocytes. 	(23, 85, 86, 103-105)
PH5CH	Lipid mediated gene transfer (lipofectin reagent) SV40 Tag	<ul style="list-style-type: none"> - Display epithelial appearance. - Express human CK and ALB protein. 	<p>Used a cell model to investigate:</p> <ul style="list-style-type: none"> - HCV infection, replication and tropism. - HCC-selective cytotoxicity of HBF-0079. - MicroRNA expression in TGF-β-induced hepatocyte EMT. - Effect of hepatitis B virus proteins on signalling mediated by members of the Toll-like/interleukin 1 superfamily. - Effect of HBV polymerase on IFN production in human hepatocytes. 	(25, 74, 75, 81, 106-113)	

Adult hepatic cell line				
Cell line	Immortalization strategy	Hepatic functionality of immortalized cells	In vitro applications	Reference
THLE	Retroviral vector	<ul style="list-style-type: none">- Display epithelial morphology.- Secrete ALB and express CK18, TF, A1AT, A2M, GST-II and very low levels of GGT at early passages. CK19 expression can only be determined at later passages. Cells are uniformly negative for AFP and factor VIII. The appearance of CK19 and decreased ALB secretion at later passages demonstrate that cells undergo dedifferentiation in culture.- Retain mRNA expression of phase II enzymes such as EH, catalase, GPX, SOD and GSTs at levels comparable to human liver, with GST-II and a mRNA as the dominant form in THLE cells or human liver, respectively.- Maintain NADPH CYP reductase expression at a lower steady-state mRNA level than in human liver.- Are able to metabolize three carcinogens, which suggests the presence and activity of CYP1A2/1A1, CYP2E1 and CYP3A4. However CYP1A2, CYP2E1, CYP3A4, CYP2A3 and CYP2D6 mRNA are not detected. The steady-state mRNA levels of CYP1A1 increase after exposure to Aroclor 1254 or Bf[α] P.	THLE and THLE-CYP cells can be used to study cellular toxicity of compounds. Used a cell model to investigate: <ul style="list-style-type: none">- HCC-selective cytotoxicity of HBF-0079.- Hepatoprotective and chemopreventive properties of phytochemicals.	(25, 93, 94, 111, 114-124)
	SV40 Tag	<ul style="list-style-type: none">➤ Overexpression of specific CYP450 gene led to the development of THLE-CYP sublines.		
TPH1	Strontium phosphate precipitation	<ul style="list-style-type: none">- Exhibit altered cell morphology resembling low-differentiated epithelial cells.	Used a cell model to investigate HCV infection and replication. Induces apoptosis of activated hepatic stellate cells.	(45, 77, 78, 125-129)
	HCV core gene	<ul style="list-style-type: none">- Express no A1AT or AFP mRNA.- Secrete ALB.- Possess G6P activity.- Reactivate telomerase immediately after senescence.		
Fetal and neonatal hepatic cell lines				
Cell line	Immortalization strategy	Hepatic functionality of immortalized cells	In vitro applications	Ref
FH-TERT	Retroviral vector	<ul style="list-style-type: none">- Express CYP450 mRNA and maintain, in contrast to passaged fetal hepatocytes, liver-enriched differentiation markers, especially C/EBPα and HNF4 as well as elevated levels of HGFR.	Used as a stroma to induce human embryonic stem cells differentiation into hematopoietic cells. Used as a cell model to investigate: <ul style="list-style-type: none">- Role of RPK4 as novel tumor suppressor in human hepatocarcinogenesis.	(34, 83, 84, 130)
	hTERT	<ul style="list-style-type: none">- Possess glycogen storage and G6P activity, in a pattern similar to primary fetal hepatocytes.- Produce urea and retain level of ALB synthesis equivalent to HepG2 cells.		

Fetal and neonatal hepatic cell lines						
Cell line	Immortalization strategy	Hepatic functionality of immortalized cells		In vitro applications	Ref	
Hc3716-hTERT		➤ Culture conditions used in these studies were designed at supporting cell proliferation; conditions have not been optimized for inducing differentiated hepatocellular functions.		<ul style="list-style-type: none">- Permissive role of β-catenin signalling in the initial phase of hepatocarcinogenesis.		
	Retroviral vector	<ul style="list-style-type: none">- Maintain normal mammalian cell morphology.- Exhibit protein expression of ALB, CK8 and CK18, but not AFP. ALB levels are higher than in control, passaged Hc3716 cells.- Possess inducible CYP3A4/7 mRNA levels.- Exhibit wild-type p53 responsiveness.		Used as <i>in vitro</i> model for predicting the side-effects of telomere-targeting drugs.	(39)	
	hTERT					
NeHepLxHT	Retroviral vector	<ul style="list-style-type: none">- Display characteristic morphology of primary fetal liver cells.- Maintain epithelial characteristics as evidenced by immunostaining for epithelial cell markers, the cytokeratins.- Possess 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 indicate the progenitor nature of the cells.		Used as a cell model to investigate: <ul style="list-style-type: none">- Role of FAT10 in promoting malignant cell transformation.- Molecular mechanism responsible for miR-224 overexpression in HCC.- Role of HCV RNA-dependent RNA polymerase in promoting liver inflammation and injury.	(14, 80, 131, 132)	
	hTERT					
OUMS-29	Lipid mediated gene transfer (lipofectin reagent)	<ul style="list-style-type: none">- Display epithelial morphology.- Maintain gene expression of ALB, ASGPR, bil-UGT, GS, GST-π, HBCF-X, Ahr and Amt.- Secrete ALB, AFP, TF, A1AT and APO A-1.- Possess inducible CYP1A1/2 mRNA levels and activity.		Used as a cell model to investigate: <ul style="list-style-type: none">- Role T-cell factor 4 isoforms in promoting hepatic tumorigenicity.- Effect of bile acid species on hepatocyte apoptosis induced by PPARgamma ligands.- Effect of hydrogen peroxide on hepatic pigment epithelium-derived factor levels.- Both morphologic and functional alterations in the mitochondria of troglitazone-treated hepatocytes.	(17, 49, 62, 133-136)	
	SV40 Tag		➤ Overexpression of HNF4 α 2 led to development of OUMS-29/H-11 cell line with increased liver-specific gene expression, such as A1AT, apolipoproteins, HBCF-X and HNF1 α .			

Table 2
Overview of conditional immortalization strategies, hepatic functionality and in vitro applications of growth-controlled human adult and fetal hepatic cell lines

(A1AT, α 1-antitrypsin; AFP, α -fetoprotein; ALB, albumin; ASGP(R), asialoglycoprotein (receptor); Bmi-1, B lymphoma Mo-ML V insertion region 1 homolog; C/EBP, Ccaat-enhancer-binding protein; CD, cluster of differentiation; CK, cytochrome P450; GS, glutamine synthetase; GST, glutathione S-transferase; HBCF, human blood coagulation factor; HNF, hepatocyte nuclear factor; hTERT, human telomerase reverse transcriptase; mRNA, messenger ribonucleic acid; PT, prothrombin; SV40 Tag, simian virus 40 large T antigen; TF, transferrin; (bil-)UGT, (bilirubin-)uridinediphosphate-glucuronosyltransferase).

Adult hepatic cell line		Hepatic functionality of conditional immortalized cells	In vitro applications	Reference
Cell line	Immortalization strategy			
16T-3	Retroviral vector	Reverted 16T-3 cells:		
	hTERT	<ul style="list-style-type: none"> Show enhanced mRNA levels of transcriptional factors, C/EBPα and HNF4α as well as increased mRNA expression of hepatocyte-specific genes, including ALB, GST-II, HBCF-X, bil-UGT, CYP3A4, GS and ASGPR. 		
	Tamoxifen-mediated self-excision (Cre-LoxP)	<ul style="list-style-type: none"> Possess increased ALB production and lidocaine metabolism, though at lower levels than normal human hepatocytes. 		(43)
HepLi-4	Retroviral vector	Reverted HepLi-4 cells:		
	SV40 Tag	<ul style="list-style-type: none"> Express similar GS and somewhat lower UGT1A1 mRNA levels than adult human liver. ALB and GST-II mRNA levels are extremely lower or higher, respectively, compared to the human liver. This indicates that HepLi-4 cells are not fully differentiated after reversion. 		
	Tamoxifen-mediated self-excision (Cre-LoxP)			(26)
HLTC-7/-11/-15/-17/-19	Retroviral vector	<ul style="list-style-type: none"> Grow as islands or sheets of cuboidal cells (HLTC-17) or display a more dispersed cuboidal-elongated morphology (HLTC-7/-11/-15/-19). 		
	SV40 Tag	<ul style="list-style-type: none"> Secrete fibrinogen at fairly constant rate in all tested cell lines at permissive (33,5°C) and non-permissive (39,5°C) temperature. 		
	Temperature-based regulation	<ul style="list-style-type: none"> Exhibit no ALB, AFP, A1AGP or secretion in any cell line at both temperatures. Cell lines HLTC-7, -15 and -19, produce A1AT at permissive temperature. However, at non-permissive temperature the secretion of A1AT is upregulated or become detectable in all the cell lines. Cell lines HLTC-17 and -11 possess no CYP activity at any temperature even after induction and stain positive for ALB, CK18, CK7, CK19 and vimentin, but negative for CK8, with almost identical patterns at both temperatures. <p>➤ The results indicate progressive phenotypic instability and loss of differentiated functions. Conversion to the non-permissive temperature does only allow significant expression of a limited repertoire of differentiated functions by the immortalized human hepatocytes.</p>		(28)

Adult hepatic cell line				
Cell line	Immortalization strategy	Hepatic functionality of conditional immortalized cells	In vitro applications	Reference
IHH10.(3)/12	Lentiviral vector	<ul style="list-style-type: none">- Display morphology reminiscent of differentiated hepatocytes.- Express ALB, A1AT, ASGPR and CYP450 mRNA levels.- Secrete liver-specific proteins, ALB and fibrinogen, at levels similar to Huh-7 cells but lower than primary hepatocytes. The IHH12 cell line do only produce fibrinogen after de-immortalization, suggesting the acquirement of a higher differentiation status in this setting. However, Cre-recombinase treatment of IHH12 cells does not significantly improve the production of ALB.- Possess inducible CYP1A1/2 activity.	A novel <i>in vitro</i> model to investigate the mechanisms and consequences of lipid accumulation in hepatocytes, independently of insulin resistance.	(19, 87)
	SV40 Tag + hTERT (IHH10) or SV40 Tag + hTERT + Bmi-1 (IHH12)			
	Recombinase- based control (Cre-LoxP)			
NKNT-3	Retroviral vector	<ul style="list-style-type: none">- Display morphological characteristics of liver parenchymal cells and look more differentiated after reversion.- Express bil-UGT, GS and GST-II mRNA levels, which increased substantially after reversion. Contradicting results are published regarding expression of ALB and HBCF-X mRNA levels. One paper demonstrates that ALB and HBCF-X mRNA are newly introduced in the reverted cells whereas several other papers already report expression of these genes and ASGPR mRNA in non-reverted cells. Nevertheless, although reversion does stimulate differentiation, mRNA levels of ALB, A1AT and TF were maximally 0.1% of primary human hepatocytes. <p>➤ Additional experiments reveal that introduction of p21 into human immortalized hepatocytes can increase ALB expression and induce a differentiated morphology.</p> <p>➤ Co-cultivation with immortalized hepatic stellate cells increases urea synthesis and protein expression of CYP3A4/2C9.</p>	Used in combination with HCV like particles as a model system for studying viral binding and entry.	(6, 29,52, 71, 79)
	SV40 Tag			
	Recombinase- based control (Cre-loxP)			
YOCK-13	Retroviral vector	<ul style="list-style-type: none">- Display morphological characteristics of normal human hepatocytes.- Express markers of hepatocytic differentiation including ALB, ASGPR, bil-UGT, CYP3A4, GS, GST-II, and HBCF-X. <p>➤ The YOCK-13 hepatocell line is derived from the reversible immortalized human hepatic cell line, TTNT-16-3, by co-expression of modified insulin.</p>		(42)
	hTERT			
	Tamoxifen-mediated self-excision (Cre-LoxP)			

Fetal hepatic cell lines				
Cell line	Immortalization strategy	Hepatic functionality of conditional immortalized cells		Reference
cBAL111	Lentiviral vector	<ul style="list-style-type: none">- Express relatively high mRNA levels of immature markers, GST-II and AFP, and very low mRNA levels of mature markers, ALB, A1AT and TF. Transcript levels of HNF4α increase after prolonged culturing.- Stain positive for GS, ALB, CK18, CK19, vimentin and the progenitor cell marker CD146 but display CK18 in a pattern characteristic of dedifferentiated human hepatocytes.- Produce urea and ALB, though at lower levels than mature human hepatocytes.	In vitro applications	(13)
	hTERT			
	Transcriptional regulation (Tet-on approach)			

Fetal hepatic cell lines					
Cell line	Immortalization strategy	Hepatic functionality of conditional immortalized cells		In vitro applications	Reference
		<ul style="list-style-type: none">- Retain no CYP1A2 and 3A4 activity (no elimination of lidocaine) but are able to eliminate galactose.	➤ cBAL111 cells resemble cells with progenitor characteristics rather than fully differentiated hepatocytes. However, there is a trend of increased and decreased expression of mature and immature markers, respectively, with culture time.		
HepCL	Retroviral vector	<ul style="list-style-type: none">- Display morphological characteristics of liver parenchymal cells.			
	SV40 Tag	<ul style="list-style-type: none">- Stain positive for ALB, CK18 and CK19.			
	Temperature-based regulation	<ul style="list-style-type: none">- Produce amounts of ALB and urea comparable to those of unmodified primary human fetal hepatocytes.		(27)	

Table 3
Conditional approaches for human hepatocyte immortalization (36)

(rtTA, reverse tetracycline transactivator; SV40 Tag, simian virus 40 large T antigen; TRE, tetracycline responsive element; tTA, tetracycline transactivator).

Conditional approach	Immortalization construct	Growth curve
Temperature-based regulation	Thermolabile SV40 Tag mutant
Recombinase-based control	LoxP – immortalization gene(s) – LoxP FRT – immortalization gene(s) – FRT
Transcriptional regulation	TRE- immortalization gene(s)