Development of an immortalised human hepatocyte cell line for the AMC Bio-Artificial Liver

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DEVELOPMENT OF AN IMMORTALISED HUMAN HEPATOCYTE CELL LINE FOR THE AMC BIO-ARTIFICIAL LIVER

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Table of Contents

Chapter 1	General introduction and outline of the thesis	page 9
Chapter 2	Assessment of *in vitro* applicability of reversibly immortalised NKNT-3 cells and clonal derivatives	page 33
Chapter 3	*In vitro* functionality of human fetal liver cells and clonal derivatives under proliferative conditions	page 53
Chapter 4	Novel immortalised human fetal liver cell line, cBAL111, has the potential to fully differentiate into functional hepatocytes	page 75
Chapter 5	The effect of culture time on the *in vitro* hepatic functionality of the immortalised human fetal liver cell line cBAL111	page 95
Chapter 6	Increased reproducibility of quantitative reverse transcriptase-PCR	page 117
Chapter 7	Summary and general discussion	page 127
	Nederlandse samenvatting	page 133
	Dankwoord	page 139

Chapter 1

General introduction and outline of the thesis

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General introduction

Summary

Acute liver failure (ALF) is a serious disease that is only effectively treated by orthotopic liver transplantation. However, the shortage of liver donors and the mortality, morbidity and life-long immunosuppressive therapy related with liver transplantation have urged the development of complementary or alternative therapies. The search for these therapies is impaired by the high variability in etiology and pathogenesis of ALF. Nevertheless, bioartificial livers (BALs) have shown to be effective in supporting the liver function in animals with ALF. Freshly isolated or cryopreserved porcine or human hepatocytes have been most frequently used as biocomponent in clinically applied BALs. Phase 1 studies of all biocomponent modalities showed safety, feasibility, and improvement of patient's biochemical, neurological, and hemodynamic parameters. However, the xenotransplantation-related disadvantages of porcine cells and the shortage of primary human hepatocytes have urged the exploration of alternative biocomponents.

The future lies in the development of one or more human hepatocyte cell lines, which will have minimal immunogenicity, no risk of xeno-zoonosis, and the requested functionality and availability. Primary sources for the development of a human hepatocyte cell line are liver-tumour-derived cell lines, immortalised mature or fetal hepatocytes and stem cells. However, in all cell types tested so far, the *in vitro* differentiation cannot be stimulated to such an extent that their functionality reaches that of primary human hepatocytes and a pilot-controlled clinical trial with C3A cells in ALF patients did not show significant improvement of survival. More insight in differentiation-promoting factors and the influence of matrix and co-culture conditions to optimize the functionality of the human hepatocyte cell lines is necessary.

1. Liver support systems for Acute Liver Failure

1.1 Acute Liver Failure

The liver performs many different functions to ensure blood homeostasis: hepatocytes synthesize various proteins, including clotting factors; they produce bile and regulate carbohydrate, fat and protein metabolism; they detoxify ammonia and many other substances, including drugs (1). When liver function fails in acute liver failure (ALF), all these processes are disturbed. ALF is therefore a severe liver disease with mortality between 60 and 90%, depending on the cause.

ALF can develop as a consequence of many different etiologies, like viral hepatitis, acetaminophen or other drug intoxication, metabolic diseases like Wilson's disease, or primary non-functioning graft after liver transplantation.

ALF patients most often present with jaundice, which is caused by the accumulation of serum bilirubin, combined with anorexia, nausea, vomiting and abdominal pain. At the time of presentation, most of the liver damage has already taken place. ALF progresses with the development of coagulopathy, cerebral oedema resulting in drowsiness and progressing to coma, renal impairment, hydrodynamic instability, increased susceptibility to infection and multi-organ failure (1). Each subgroup of ALF patients shows specific complications. For example, empirical evidence shows that 30% of the patients with acetaminophen induced ALF develop acidosis, where in other etiologies only 5% of the patients show pH disturbance; the underlying mechanism is unclear (2). The pathogenesis of cerebral oedema, which accounts for most deaths, is still heavily debated. A combination of vasogenic and cytotoxic mechanisms is probably involved and increased permeability of the blood-brain barrier, accumulation of astrocytic glutamine, disturbance of the neuronal neurotransmitter balance as well as the accumulation of inflammatory cytokines may play a role (2).

The only life-saving therapy, orthotopic liver transplantation (OLT), is limited by the scarcity of donor livers and is associated with a relatively high mortality. Adam et al. reported a survival of 66% and 61% at 5 and 8 years, respectively, of 22,089 patients who had undergone OLT procedures during 1988–1997 in 103 active centers (3). The frequency of death declined from the first week onwards and 65% of deaths occurred in the first 6 months. In addition to mortality, OLT has considerable morbidity and transplanted patients need lifelong immunosuppressive therapy. Further medical treatment of ALF patients is mainly supportive, including mechanical ventilation and aggressive daily microbiological surveillance until recovery or until OLT is available. To bridge patients with ALF more successfully to OLT or even to regeneration of the native liver, a diversity of liver support systems has been developed.

1.2 Liver support systems

The first liver support therapies, already in the 1950s, were artificial devices that mainly focused on the detoxifying function of the liver (4-6). However, the limited function of these systems could not elongate life span, although neurological status improved. Currently, the molecular adsorbent recirculating system (MARS) is the only artificial system that did show improved survival in a controlled trial in a subgroup of patients with hepatorenal syndrome (7). One of the main reasons for the limited success of artificial devices, is most likely the non-specific absorption, e.g. the MARS cannot distinguish harmful molecules from substances that might be beneficial to recovery of the liver, like hepatocyte growth factor (HGF) (8). In addition, systems based on absorption do not support the synthetic and homeostatic functions of the liver.

Early liver supporting therapies with a biological approach include plasma exchange, ex-vivo perfusion of animal livers or rejected donor liver and even cross-hemodialysis, in which the blood of an ALF patient is dialysed against the blood of a healthy person or even of healthy animals, like dogs (reviewed by (9)). All these biological approaches had a positive effect on the patient's condition, but none of them became generally accepted, because they were too harmful for the necessary donors or because they were too complex; for the cross-hemodialysis of one patient four dogs were needed. However, these experiments laid the fundaments for the bioartificial liver (BAL) support systems that are currently developed.

Bioartificial liver systems

Ten BAL systems have been clinically applied so far (reviewed by (10)). The principal components of these BAL systems are bioreactors filled with liver cells. In most of these bioreactors the biocomponent consists of porcine hepatocytes, either freshly isolated or cryopreserved (10). Only the ELAD system uses tumour derived human liver cells, the C3A cell line, which will be discussed in paragraph 3.1. The bioreactors are included in the patients' circulation and either blood or plasma is perfused through the bioreactor. A semi-permeable membrane separates the cells from the blood or plasma; the pore size of these membranes varies between the different systems from 70 to 400 kD cut-off. The AMC-BAL is an exception; in this device the patient's plasma contacts the hepatocytes in the bioreactor without the interference of any membrane resulting in optimal mass transfer.

A number of BAL devices significantly prolonged survival time in animal models of ALF (10). Phase 1 studies of BALs charged with either porcine or human hepatocytes showed safety, feasibility, and improvement of patients' biochemical, neurological, and hemodynamic parameters. However, significant improvement of survival of patients with ALF was not shown in controlled trials by intention-to-treat analysis (reviewed by (10, 11)). Using porcine hepatocytes inside BALs is hampered by potential immunologic problems:

continuous or repeated use of these xenogeneic cells might introduce anaphylactic reaction after 1 week (12-14). In addition, for many Western countries the risk of transmission of zoonosis, including the porcine endogenous retrovirus (PERV), is still a reason to reject this form of xenotransplantation (15). Potential physiological incompatibilities and ethical considerations are additional reasons to replace the animal cells. Therefore, future BAL application ideally will be based on human liver cells as biocomponent. Moreover, the utilization of human cells, leading to no or low immunological response, enables the treatment of patients with chronic liver failure in addition to the patients with ALF. An intermittent treatment of these patients on a frequent base will increase the quality of life of an estimated 50,000 patients in the United States with chronic liver failure yearly.

Human hepatocytes in BAL application.

Primary human hepatocytes are, however, scarce and undergo maximally one to two cell divisions *in vitro*, and therefore cannot be efficiently expanded. Some transplantation centers have possibilities to isolate primary human hepatocytes from discarded liver not suitable for transplantation. Most of these livers are discarded because of high fat content (steatotic livers), but hepatocyte isolation with sufficient yield and acceptable vitality is sometimes possible (16, 17).

Twelve patients (3 patients with ALF, 3 with primary non-function after OLT, and 6 patients with acute on chronic liver failure) have been treated using a BAL device loaded with primary human hepatocytes (Sauer, personal communication (18, 19)). This phase 1 study showed safety of the procedure and life-supporting capacity. The only other clinical study performed with primary human hepatocytes was a single treatment of more than 100 hours, during which clinical parameters improved (16). However, availability of primary hepatocytes at the right time, when an ALF patient presents him- or herself, is still an important drawback of this source of cells, especially as long as cryopreservation of human liver cells is insufficient.

Because the availability of mature human hepatocytes is insufficient for large-scale BAL applications, many researchers are exploring alternative cell sources for BAL systems. In this respect, cell lines derived from human liver tumours and *in vitro* immortalised liver cells as well as stem cells from different origins have been investigated.

2. Criteria for human hepatocyte cell lines

When evaluating alternative cell sources for BAL applications, three main aspects should be considered: availability, functionality and safety (Table 1).

2.1 Availability

Cryopreservation techniques are insufficient to overcome the issue of the unpredictable availability of human liver; after thawing and warming, cryopreserved cells show a significant loss of viability and lose significant capacity of attachment to matrices. To support an ALF patient, a BAL system should be charged with at least an equivalent of 10 billion fully functional primary hepatocytes (10). In case of an expandable cell mass, this means that 34-35 population doublings are necessary when starting with one cell (20). A stable cell source that can be expanded indefinitely in a laboratory will be most convenient for large-scale applications, because it ensures continuous cell supply with a standardized quality.

***Table 1**: Requirements of alternative cell sources for BAL application*

	Characteristics necessary for BAL application	**Remarks**
Availability	Equal to or more than10^{10} fully differentiated hepatocytes per BAL	Cell source with indefinite proliferation capacity is preferred
Hepatic function	Ammonia elimination Detoxification of drugs/toxic compounds Carbohydrate homeostasis Protein synthesis	Specific functions can be increased by genetic modification
Safety	Free of pathogens Non-immunogenic Non-tumorigenic or no tumour transmigration	Suicide genes can be introduced in cell lines. Filters in BAL circulation can prevent cell transmigration

2.2 Functionality

As described in paragraph 1.1, it is very difficult to list the functional demands for the biocomponent of BAL systems, because of the variation in the ALF patient population and the insufficient knowledge of the pathogenesis of hepatic encephalopathy.

Some biosynthetic functions, for example synthesis of coagulation factors and albumin, do not have to be performed by the BAL, since those proteins can be supplied to the patient by intravenous infusion. However, the existence of a biosynthetic pathway that plays an important but yet unknown role in ALF treatment and for liver regeneration, cannot be excluded. A function that is indispensable for artificial liver support systems is the detoxification of ammonia because hyperammonemia causes major complications in the majority of ALF patients (21). In addition, biotransforming properties as well as homeostatic

functions might be required. Hepatic functions that are generally thought to be necessary for BAL applications are listed in Table 1.

Freshly isolated mature hepatocytes are the only cells that proved to support liver function and to prolong survival time in ALF animal models and therefore are the functional standard. The functionality of alternative cell sources should be compared with that of primary hepatocytes, to critically assess the potential of these cells for BAL systems. Because the functionality of hepatocytes and hepatic cell lines *in vitro* drastically depend on culture conditions, isolated mature human hepatocytes should be included as reference in all investigations of alternative cell sources. Many studies lack this comparison, which impairs critical assessment and comparison between investigations.

Importantly, functionality should be stable once the cell source, potentially after a differentiation regime, is at operating level in the BAL system. Utilization of clonogenic cell lines and minimization of expansion will maximize the stability of cell mass compositions. Loss of functionality per cell is most critical during treatment of an ALF patient as exposure to plasma of ALF patients significantly reduces most hepatic functions, e.g. capacity to eliminate ammonia and cytochrome P450 activity, although viability of the hepatocytes may remain (22). The mechanism underlying this hepatocyte specific downregulation is not yet revealed, but may be due to the accumulation of cytokines or toxic metabolites.

2.3 Safety

Alternative cell sources for BAL applications should obviously not harm the patient and should be free of hepatitis viruses and HIV. In addition, the cell source should preferably be non-tumorigenic. In an experiment in which HepG2 cells were cultured in a BAL for 60 days, a large number of tumour nodules were found in the extracapillary compartment, that is in direct contact with the patient, at termination of the experiment (23). It was unclear whether the HepG2 cells had passed the hollow-fiber membrane during the experiment or whether the cells were accidentally inoculated in the extracapillary compartment during BAL assemblage. However, this example stresses the importance of this safety issue.

If a tumour derived cell line cannot be avoided, the hardware of BAL systems should ensure the encapsulation of the cells. Additional safety can be included by the introduction of a suicide gene into the cells, like the herpes simplex virus thymidine kinase (HSV-TK) gene, which results in sensitivity for ganciclovir (24). In the case of suspected migration of tumour cells to the patient's body, a ganciclovir treatment of the patient should eliminate the tumour cells.

3. Alternative cell sources for BAL application

3.1 Tumour derived cell lines

Over the last four decades, a number of hepatoma or hepatoblastoma derived cell lines with excellent growth characteristics have been reported. A summary is given in Table 2.

One of the first available well-differentiated and therefore most investigated human hepatoma cell lines is the HepG2 cell line. HepG2 cells are well known for their albumin-synthesizing capacity, but the ammonia detoxification and mixed function oxidase activity is very poor (23; 25). The absence of a functional urea cycle is the result of a deficiency for both arginase and ornithine transcarbamylase (26). Originating from a human liver tumour, this cell line should be applied in BAL systems with care. As a result of selection for albumin and AFP excretion, strong contact inhibition and the ability to grow in glucose deficient medium, the C3A cell line has been obtained from the original HepG2 cell population. This clonal derivative has been used in the extracorporeal liver assist device (ELAD) system in a pilot-controlled clinical trial in patients with ALF in the United Kingdom (27). However, no significant effect on survival was observed. Currently, a new trial is going on in China, which will also use C3A cells (see website of Vital Therapies; www.vitaltherapies.com).

Another cell line has been isolated from a liver tumour associated with chronic hepatitis C (HCV) (28). The cell line is considered to originate from ductular proliferation associated with long-term HCV infection, although the cells are tested HCV negative and exhibit contact inhibition. This cell line, designated HepaRG, co-expressed hepatocyte and bile-duct markers. At high cell density and in the presence of dimethylsulfoxide (DMSO) and epidermal growth factor (EGF) this cell progressively acquired polarized hepatic phenotype with expression of e.g. albumin and hepatocyte-specific (HP-1) antigen (29). The cells expressed some cytochrome P450 (CYP) genes, specifically CYP 3A4 and CYP 2B6, up to the level of mature primary hepatocytes, however other CYP genes were expressed at levels less than 5 % (30). Although these cells clearly have potential in xenobiotic metabolism, their overall hepatic differentiation is probably not optimal, since the cells continued to express cytokeratin 19 (CK19), a bile-duct marker, and CK14, a progenitor marker, after induction of hepatic differentiation (29).

The insufficient level of hepatic differentiation of tumour derived cell lines may be due to the upregulation of tumour-like properties necessary for proliferation at the expense of other functions. At present it should be concluded that hepatoma or hepatocellular carcinoma-derived liver cell lines need drastic improvement of liver-specific function and safety aspects, before they can be considered suitable for BAL support.

3.2 *In vitro* immortalised cell lines

With improved techniques for genetic modifications, many *in vitro* immortalised cell lines have been developed that have sufficient growth potential. Genes that prevent terminal growth arrest have been introduced in isolated hepatocytes, sometimes in an inducible system. The majority of cell lines has been developed by the introduction of Simian virus large T antigen (SV40T). This protein is an oncogenic factor (31) which neutralises the cell cycle inhibitors p53 and the retinoblastoma protein, stimulating cell cycle progression. Another immortalisation strategy is the stabilisation of telomeres by the introduction of human telomerase reverse transcriptase (hTERT), which prevents the erosion of telomeres until the critical length that induces growth arrest (32; 33).

The advantage of *in vitro* immortalised cells over tumour derived cell lines is that the immortalising events are known, which allows more control over the immortalisation process. Immortalising events which are less drastic than those resulting in undifferentiated tumours can be selected. However, additional spontaneous mutations contributing to the immortalisation process cannot be excluded. The initial growth potential of the cells used for immortalisation, defines the required immortalisation strategy and influences the functional outcome of the immortalisation.

Immortalised Mature Human Hepatocytes

Multiple immortalised mature human hepatocytes have been introduced. A selection of the best described cell lines is given in Table 3. One of the most promising cell lines, the reversibly immortalised human liver cell line NKNT3, was introduced in 2000 (34). Adult human hepatocytes were immortalised by retroviral introduction of the SV40T gene, which was located between two loxP sequences. These loxP sequences enabled excision of the SV40T gene by Cre recombinase. Adenoviral introduction of Cre recombinase led to removal of the immortalising gene, which stopped proliferation. In addition, cre–lox recombination led to activation of a neomycin-resistant gene that allowed selection for reversion of immortalisation. After reversion, an increase in mRNA expression of liver-specific genes could be demonstrated. Transplantation of 50 million cells intraperitoneally in 90% partially hepatectomized rats showed a significant improvement of survival. However, comparison of *in vitro* functionality of NKNT-3 cells with primary mature hepatocytes was lacking and transplantation of non-reverted and reverted NKNT-3 cells was equally beneficial to the hepatectomized rats. Therefore more research on their applicability in BAL systems was required.

***Table 2**: Overview of tumour derived hepatocyte cell lines*

Characteristic / Cell line	Origin	Tumourgenicity	Function *in vitro*						Bioartificial Liver experiments	Ref.
			Albumin	AFP	AAT	Cyt. P450	Urea prod.	NH_3 elim		
HepG2	Hepatocellular carcinoma, 15 year old male.	Pos in SCID mice *	Pos	Pos	Pos	Neg 3A4	Pos		*In vitro*: primary rat hepatocytes outperformed HepG2 in all biotransformation catagories	(23) and ATCC datasheet
C3A	Clonal derivative of HepG2, selected for strong contact inhibition, high production of albumin and AFP and growth in glucose deficient medium	Neg in SCID mice	Pos	Pos	Pos	Pos 1A1			Pilot controlled clinical trial in ELAD system did not show effect on survival.	(27; 66) and ATCC
HepG2-GS	Glutamine synthetase gene introduced in HepG2	Not specified						Pos	Animal model of ALF: increased survival time compared to BAL with HepG2	(67; 68)
HepG2-GS-Cyp3A4	Glutamine synthetase and Cyp3A4 gene introduced in HepG2	Not specified				Pos 3A4		Pos	Animal model of ALF: increased survival time compared to BAL with HepG2-GS	(68)
HepaRG	Hepatocellular carcinoma, adult female	Not specified	Pos	Pos		Pos 1A2, 2B6, 2C9, 2D6, 2E1, 3A4				(69)
HuH-7	Hepatocellular carcinoma, 57 year old male	Pos in SCID mice	Pos	Pos	Pos					(70; 71)

** although the ATCC datasheet states that HepG2 does not form tumours in SCID mice, several investigators use HepG2 cells to induce tumour formation in SCID mice (72; 73).*
Abbreviations: AAT = alpha-1-antitrypsin, AFP = alpha-fetoprotein, cyt = cytochrome, elim = elimination,neg = negative, pos = positive, pRb = Retinoblastoma protein, prod = production, ref = reference, SCID =severe combined immunodeficiency, SV40 = simian virus 40, wks = weeks.

Immortalised Fetal Human Hepatocytes

More recently, human fetal liver cells are used as starting material for immortalised human hepatocyte cell lines. In contrast to adult hepatocytes, fetal hepatocytes still have the capacity to undergo multiple cell divisions *in vitro*. So, if growth capacity is sufficient, immortalisation may not even be needed to load BAL devices. Wege et al. showed that human hepatocytes isolated from 22–24 week old foetuses entered growth arrest after 30–35 population doublings, which is sufficient for BAL application (35). To warrant stability and unlimited availability of the cells, immortalisation of fetal hepatocytes and generation of cell lines is still required. Probably immortalisation is more easily established in fetal cells as compared to adult hepatocytes, since less interference in the cell cycle control is required.

Despite the proliferation capacity of fetal liver cells, again most cell lines have been immortalised by SV40T (Table 4). One of the first immortalised human fetal liver cell lines was OUMS-22 by Miyazaki et al. (36), however, the functionality of these cells was low and no additional data were reported since then. Yoon et al. immortalised human fetal liver cells by introducing SV40 large and small T (37). Butyrate treatment increased the amount of albumin secretion, cytochrome P450 activity, and urea production. However, compared to freshly isolated human liver cells, urea production in the presence of a very high ammonia concentration (20 mM) was only 1%. The human fetal liver cell line that has been studied most is OUMS-29, generated by Fukaya et al. (38), but again its functionality is low. The cells were not able to significantly improve survival of 90% hepatectomised rats after transplantation. Since then, their hepatic function *in vitro* has been improved by HNF4α introduction and culturing in a three dimensional configuration; Cyp3A4 activity became detectable.

Wege et al. reported for the first time the immortalisation of human fetal hepatocytes (FH-hTERT) by hTERT reconstitution (35). In contrast to SV40T, hTERT immortalisation does not lead to dramatic changes in the expression profile of many cell types (31; 39). FH-hTERT cells surpassed terminal growth arrest, performed more than 300 population doublings and their differentiation potential was not disrupted. They appeared to be non-tumorigenic, and *in vitro* functionality was to some extent comparable with single-time passaged fetal hepatocytes, as demonstrated for mRNA of hepatic growth factors, transcription factors, cytochrome P450, albumin, glucose-6-phosphatase, and glycogen synthesis. These cells were not compared with primary adult hepatocytes. However, the observed level of urea synthesis, up to 0.08 umol/h/million cells, is sufficient for BAL application, as defined by Tsiaoussis et al. (40). These cells are therefore very promising, but clonal derivatives should still be generated. Such an approach could yield several valuable cell lines.

***Table 3**: Overview of in vitro immortalised mature human hepatocyte cell lines.*

Charac-teristic / Cell line	Immortalisation		Tumorgenicity	Function *in vitro*						Functionality *in vivo*	Ref.
	Origin of cells	Genes		Albu-min	AFP	AAT	GST pi	Cyt. P450	Urea prod.		
Fa2N-4	12 year old female	SV40 large T antigen						Pos: 1A2, 2C9, 3A4			(74)
HepLL	25 year old male	SV40 large T antigen	Neg after 3 months in SCID mice.	Pos	Neg		Pos	Pos: 2E1	Pos		(75)
HepZ	Not specified	E2F, Cyclin D1, antisense pRb, antisense p53		Pos				Pos: 3A4			(76)
IHH10.3	37 year old female	SV40 large T, TERT	Both cell lines: Neg after 3 months in SCID mice.	Both: Pos		Both: Pos		Both cell ines: Pos: 1A1/2, 2D6, 3A4, 3A5, 4B1		Both cell lines: ALF mouse model: increased survival	(77)
IHH12	Idem	SV40 large T, TERT, Bmi1									
NKNT-3	Not specified	SV40 large T antigen (cre-loxP)	Neg after 2 months in SCID mice	Pos			Pos	Pos: 3A4, 2C9		90% PHX: increased survival	(34)
THLE-2 and THLE-3	Not specified	SV40 large T antigen		Both: Pos		Both: Pos	Both: Pos	Both cell lines Pos: 1A1, Neg: 1A2, 2A3, 2E1, 2D6, 3A4			(78)

Abbreviations: AAT = alpha-1-antitrypsin, AFP = alpha-fetoprotein, cyt = cytochrome, GST = glutathion-S-transferase, neg = negative, PHX = partial hepatectomy, pos = positive, pRb = Retinoblastoma protein, prod = production, ref = reference SCID =severe combined immunodeficiency, SV40 = simian virus 40, wks = weeks.

***Tabel 4**: Overview of immortalised human fetal liver cell lines*

Charac-terisitic / Cell line	Immortalisation		Tumorgenicity	Function *in vitro*						Functionality *in vivo*	Ref.
	Origin of cells	Genes		Albu-min	AFP	AAT	GST pi	Cyt. P450	Urea prod.		
Fetal hepatocyte	Not specified	SV40 large and small T antigens	Neg after 20 weeks in SCID mice.	Pos	Neg			Pos: 2D6, 3A3, 3A4	Pos		(36)
Fetal hepatocyte	22-24 wks. gestational age	hTERT	Neg after 15 weeks in SCID mice	Pos				Pos: 1A1, 1B1, 2B, 2E1	Pos	Hepatic differentiation in murine liver	(34)
OUMS-22	18 wks. gestational age	SV40 large and small T antigens	Neg after 6 months in SCID mice	Neg	Neg		Pos				(35)
OUMS-29	21 wks. gestational age, male	SV40 large and small T antigens		Pos	Pos	Pos		Pos: 1A1, 1A2			(37)
OUMS-29 / HSV tk		Introduction of HSV-tk suicide gene	Neg after 3 months in SCID mice.	Pos			Pos			90% PHX: increased survival, not significant	(78)
OUMS-29 / H-11		Introduction of HNF4 α, cultured in radial-flow bioreactor		Pos				Pos : 3A4			(79)

Abbreviations: AAT = alpha-1-antitrypsin, AFP = alpha-fetoprotein, cyt = cytochrome, GST = glutathion-S-transferase, HSV-tk = Herpes Simplex Virus tyrosine kinase, neg = negative, PHX = partial hepatectomy, pos = positive, prod = production, ref = reference, SCID =severe combined immunodeficiency, SV40 = simian virus 40, wks = weeks.

3.3 Stem cells

Stem cells can be defined as cells that can proliferate indefinitely in an undifferentiated state and have the potency to differentiate into multiple cell types. Stem cells from different origins are suggested to have the ability for hepatic differentiation. However, the question whether this system is sufficient for *in vitro* applications that need hepatocyte functionality, remains to be answered.

Human Hepatic Stem Cells

Attention has been drawn to the fact that hepatic stem cells play a role in liver regeneration. The liver contains intrahepatic "stem cells", which can regenerate a transit compartment of progenitor cells representing the human counterparts of oval cells in rodents (41). This reservoir of stem cells proliferates and generates lineage, only if mature hepatocyte proliferation is blocked or delayed, e.g. during liver cell damage due to hepatotoxicity. The progenitor cells concomitantly express hepatocyte and bile-duct markers and can differentiate along both lineages. Suzuki et al. have pointed to the roles of hepatocyte growth factor (HGF) and CCAAT/enhancer-binding protein (C/EBP) as possible regulators of differentiation versus proliferation in mice hepatic stem cells (42). Although Allain et al. were able to immortalise a primate (cynomolgus monkey) bipotent epithelial liver stem cell by SV40T gene, until recently no human progenitor cells could be sufficiently expanded *in vitro*; and since their quantity is extremely low, these cells cannot yet be considered as a potential cell source for BAL application (43).

Human Hematopoietic Stem Cells

In 1999 and 2000, it was reported that multipotent adult progenitor cells from bone marrow of mice and humans were able to transdifferentiate into hepatocyte-like cells *in vivo* (44-47). In the following years also, *in vitro* hepatic differentiation of bone-marrow cells was described (48; 49). However, in 2003, evidence accumulated that the observed liver repopulation with bone-marrow-derived cells could be mainly attributed to fusion between the donor-derived bone-marrow cells and the host hepatocytes (25; 50). By transplantation experiments in the fumarylacetoacetate-hydrolase-deficient mouse model, Willenbring et al. identified the subpopulation of bone-marrow cells producing functional hepatocytes by fusion as myelomonocytic cells, such as macrophages (51). On the other hand the possibility of hematopoietic stem cells transdifferentiating into hepatocytes cannot be completely excluded. For instance, Newsome et al. showed that human cord-blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion (52). Yet, at present, both transdifferentiation and cell fusion have too low capacity to consider hematopoietic cells as useful cell source for BAL systems.

Human Embryonic Stem Cells

Embryonic stem (ES) cells have the potential to differentiate *in vitro* along the hepatic lineage; however, the undifferentiating propagation of the stem cells as well as an efficient hepatic differentiation are major hurdles to take for BAL application. Initially human ES (hES) cells required a feeder layer of mouse embryonic fibroblasts to maintain pluripotency and unlimited growth capacity. To allow large expansion of cells, feeder-free culturing techniques utilizing Matrigel and conditioned medium have been developed (53; 54), which offer opportunities to culturing within a BAL device. However, the use of Matrigel, which derives from mouse sarcoma, is associated with the risk of introducing murine viruses in the human cell line-a risk that should be avoided. Sato et al. showed that activation of the canonical Wnt pathway is sufficient to maintain self-renewal of hES cells by using 6-bromoindirubin-3 oxime, a specific inhibitor of glycogen synthase kinase 3. If unlimited propagation of hES cells becomes possible, the next hurdle is even a greater challenge-differentiation of hES cells into hepatocytes (55). At present there are no differentiation strategies to efficiently produce hepatocytes from human ES cells, although some successes have been reported by applying β nerve growth factor (β-NGF) and hepatocyte growth factor (HGF) (56) and modified culture media (57).

From differentiation studies on mouse ES cells, it is well known that cytokines, growth factors, culture conditions, extracellular matrix, and cell-cell contacts are critical in directing endodermal differentiation. Recent studies showed that mES cocultured with immortalised stellate cells, endothelial cells and cholangiocytes on an unwoven polytetrafluoroethylene cloth combined with treatment with Fibroblast Growth Factor-2, DMSO, HGF and dexamethasone, resulted in functional hepatocytes that, when placed in an intracorporeal BAL module, saved 90% hepatotectomized rats (58). In addition, treatment with DMSO and sodiumbutyrate combined with culturing on a collagen coated surface (59) and coculturing mouse ES with Thy-1 positive fetal liver cells (60) stimulated differentiation in the hepatic direction. However, the relevance of results obtained with mouse ES cells for human ES cells remains to be investigated.

4. Discussion and conclusions

The development of proliferating human hepatocyte cell lines is crucial for the success of BAL treatment. It is clear that the ideal hepatocyte cell line for BAL support is still not available, although progress has been made in elucidating fundamental aspects of liver cell proliferation and differentiation. To date one of the most promising strategies seems to be the immortalisation of fetal hepatocytes by overexpressing hTERT (35).

Probably the ideal human hepatocyte cell line for BAL application will be extremely difficult to acquire, given the conditions to which the cells should answer: unlimited lifespan, safety, and high proliferation capacity combined with full hepatic functionality. Concerning the first two conditions most progress has been made in the last years. Safety of the cells is considered to be achievable by using non-tumorigenic cells and incorporation of adequate barriers between the BAL device and the patient. Another option is the introduction of suicide genes into the cells which allows selecting against cells that have escaped from the device by treatment of the patient with a prodrug (34; 61). However, it is clear that the hepatic differentiation of the proliferating cells should be drastically increased, before clinical application in a BAL can be pursued.

Paracrine factors, extracellular matrix and cell-cell interactions with neighbouring hepatocytes and non-parenchymal cells form a complex network of signals that are critical for hepatic differentiation *in vivo*. Details about these signals should be elucidated, to apply them for hepatic differentiation *in vitro*. In the setting of BAL devices, the 3D configuration of the cell culture and shear forces of medium that is perfused may stimulate hepatic differentiation and organization (62-64). Even though hepatocytes *in vivo* can proliferate and perform full hepatic function during liver regeneration, different experiments suggest that proliferation and hepatic differentiation *in vitro* are mutually exclusive (29; 65). These findings resulted in the method of reversible immortalisation in which the cell line can be switched from a proliferative mode to a differentiation mode by excision of the immortalising gene. Although this attractive method does induce hepatic differentiation when proliferation is inhibited, this induction of differentiation should be considerable to ensure sufficient hepatic function.

In conclusion, the present human cells and cell lines are not well described or suitable for BAL application, primarily due to insufficient functionality. Therefore, existing cell lines should be tested more thoroughly and more cell lines should be generated with limited interference in the cell cycle control of the cells and with preservation of their potential to hepatic differentiation. In addition, the mechanisms of hepatic differentiation with the translation to *in vitro* conditions should be subject for future research.

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Outline of this thesis

The aim of the research described in this thesis was to develop a cell source that can be used to replace mature porcine hepatocytes in BAL applications. Therefore we started out with the investigation of an established human hepatocyte cell line, NKNT-3, that proved to function as hepatocyte *in vivo*, which is described in **Chapter 2**.

In **Chapter 3** the possibility of using human fetal liver cells for *in vitro* hepatic functionality was investigated. The clones that were selected during these experiments were exposed to an immortalisation protocol. This process and the resulting immortalised cell line, entitled cBAL111, are described in **Chapter 4**. The cell line cBAL111 showed to have full differentiation capacity *in vivo*, but hepatic functionality *in vitro*, still needed improvement. **Chapter 5** describes the culture conditions that contribute to this improvement.

During all these studies, real-time RT-PCR was an often used method to quantify mRNA levels of relevant genes. The modifications that were made to this method to ensure reproducibility are described in **Chapter 6** of this thesis.

Chapter 2

Assessment of *in vitro* applicability of reversibly immortalised NKNT-3 cells and clonal derivatives

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Abstract

In vitro applications of human hepatocytes, such as bioartificial livers and toxicity assays, require thoroughly testing of human cell lines prior to using them as alternative cell sources. The reversibly immortalised NKNT-3 cell line was reported to show clear *in vivo* functionality. Here, NKNT-3 cells were tested for their *in vitro* applicability. Low-passage (P2) and high-passage (P28) NKNT-3 cells and clonal derivatives were characterized for reversion of immortalisation, heterogeneity, and hepatic functionality. Reversion with reduced expression of immortalising agent could be established. However, during culturing the cells lost the capacity to be selected for completed reversion. The phenotypic instability is probably associated with heterogeneity in the culture, as clonal derivatives of P2 cells varied in morphology, growth, and reversion characteristics. The mRNA levels of genes related with hepatic differentiation increased 4–20-fold after reversion. However, the levels never exceeded 0.1% of that detected in liver and no urea production nor ammonia elimination was detected. Additionally, activities of different cytochrome P450s were limited. In conclusion, the NKNT-3 culture is heterogeneous and unstable and the *in vitro* functionality is relatively low. These findings emphasize that *in vivo* testing of hepatic cell lines is little informative for predicting their value for *in vitro* applications.

Keywords: Hepatocyte; Immortalisation; Telomerase; SV40 large T antigen; Liver

Introduction

The *in vitro* use of human hepatocytes for *e.g.* bioartificial livers, toxicity assays and investigations of liver diseases and therapies has been hampered by the scarcity of human liver material and the lack of *in vitro* proliferation capacity of primary hepatocytes (1-3). As an alternative, hepatoblastoma or hepatoma cell lines and immortalised cell lines have been developed (4). Although these cell lines exhibit excellent proliferation characteristics, most of their differentiated hepatic functions, such as ammonia elimination or cytochrome P450 (CYP) activity, are limited (3-5). Conversely, hepatic proteins that are expressed in the immature liver, such as glutathione S-transferase π (GSTπ) or alpha-fetoprotein (AFP), are often elevated (6,7). Moreover, these cell lines may display malignant transformation, as a consequence of their origin or immortalising genes and mutations (8). Taken together, these cell lines are not suitable for most applications requiring cells displaying a primary human hepatocyte phenotype.

To counteract the putative tumorigenicity associated with immortalising gene expression, two conditionally immortalised human hepatocyte cell lines, NKNT-3 and TTNT-16-3, have been developed (9-10). In these cell lines the processes of differentiation and proliferation, that are mutually exclusive in most mature cells (11), are separated, allowing cell lines to differentiate when sufficient cell mass has been obtained. The NKNT-3 cells have been generated by immortalising human hepatocytes through the introduction of a *Simian Virus 40 Large Tumor Antigen* (*SV40T*) expressing construct under Cre/loxP control. A neomycin resistance (Neo^R) gene that is only active after Cre/Lox recombination, was incorporated in the construct, allowing selection for completion of Cre/Lox recombination after transduction of the Cre recombinase expressing adenovirus AxCANCre (12). The non-reverted NKNT-3 cells expressed low hepatic functions *in vitro*, however AxCANCre transduction followed by neo^R selection yielded reverted cells with substantially increased mRNA levels of liver-specific genes. This was also previously observed in a rat hepatocyte cell line similarly reversibly immortalised and analysed in a non-quantitative way (13). Transplantation of the reverted NKNT-3 cells into rats that had undergone 90% hepatectomy significantly increased survival rate and liver-specific parameters (9).

To assess whether the NKNT-3 cells meet the demands for *in vitro* applications, their liver functions should be comparable to that of primary human hepatocytes. A comparison of the *in vitro* liver functions of NKNT-3 cells with primary human hepatocytes is lacking however, as is most often the case when hepatic cell lines are presented. Here we report a further characterization of NKNT-3 cells and a comparison with primary human hepatocytes or human liver material for assessment of their *in vitro* applicability.

Materials and Methods

Cells, liver tissue and culture conditions

NKNT-3 cells were kindly delivered by prof. I. Fox, University of Nebraska, USA. The NKNT-3 cells were cultured in 6-well primaria culture plates (Falcon) and in 75 cm^3 culture flasks in CS-C complete serum free medium (Cell Systems Corporation) with 0.2 mg/ml hygromycin B and 1 U/ml penicillin/streptomycin and passaged with a split ratio of 1:5 according to instructions for CS-C medium. The medium was changed every 2-3 days and cultures were maintained at 37ºC in a humidified atmosphere (95% air, 5% CO_2).

We obtained 3.7-44 gram non-tumor parts of livers from eight patients who received hepatic resections. The procedure was in accordance with the ethical standards of the institutional committee on human experimentation and the Helsinki Declaration of 1975. The resection sample was transferred on ice and samples of 0.2-0.5 gram were sliced off and deep-frozen in liquid nitrogen within 15 minutes after the resection had taken place to serve as control material for mRNA quantification experiments.

Subsequently, hepatocyte isolation from the remains of the liver sample was performed by an adaptation of the calcium two-step collagenase perfusion technique (14). The liver sample was flushed at room temperature through the veins using cold Ringer glucose solution (NPBI) containing 10 U/ml heparin (Leo Pharma). Perfusions were carried out by repeated injections using a syringe. Hundred ml of calcium-free solution (137 mM NaCl (Merck), 2.7 mM KCl (Merck), 0.5 mM EGTA (Boehringer Mannheim), 10 mM HEPES (Roche), 10mM glucose (Merck), 0.7mM Na_2HPO_4 (Merck), 100.000 IU/l penicillin-G (Yamanouchi), 40 mg/l gentamycine (BioWhittaker), 100 mg/l vancomycin (Eli Lilly), 2 mg/l fluconazol (Diflucan, Pfizer Inc.), pH 7.5) was subsequently perfused at 37 ˚C for 10 min. In the next step the liver sample was perfused at 37ºC for 15 min with 100 ml digestion solution (137 mM NaCl, 2.68 mM KCl, 5 mM $CaCl_2$ (Merck), 10 mM HEPES, 10 mM glucose, 0.7 mM Na_2HPO_4, 0.4 g/l collagenase P (Roche), 100.000 IU/l penicillin-G, 40 mg/l gentamycine, 100 mg/l vancomycin, 2 mg/l fluconazol, pH 7.5). The liver sample was then sliced in small 2 mm^3 pieces and a first fraction of liver cells was harvested by filtration through surgical gauze and kept on ice. Remaining liver pieces were gently shaken in digestion solution at 37 ˚C for 15 min. The liver pieces were again sliced and the second fraction of liver cells was harvested by filtration as described. The combined fractions of liver cells were washed three times with WE culture medium by centrifugation at 4º C and 50xg for 3 min followed by resuspension in culture medium. WE culture medium was based on Williams' E medium (BioWhittaker) supplemented with 4% (v/v) heat inactivated fetal bovine serum (FBS) (BioWhittaker), 2 mM glutamine (BioWhittaker), 1 µM dexamethasone (Centrafarm), 20 mU/ml insulin (Novo Nordisk), 2 mM ornithine (Sigma-Aldrich), and penicilline/streptomycine/fungizone mix (Bio-

Whittaker). The viability and yield of the cell isolate was determined by trypan blue exclusion test and using a Bürker Bright line cytometer (Optik Labor).

The hepatocytes were seeded in 6-well primaria culture plates at a density of 1.10^6 viable cells/well and cultured in WE medium. Four hours after seeding the medium was replaced by fresh WE medium, CS-C complete serum free medium with 1U/ml penicillin/streptomycin or the chemically defined ISE-RPMI medium (15).

Generation of NKNT-3 clonal derivatives

Low-passage NKNT-3 cells were cloned by limiting dilution. Clonal derivative cBAL58 was generated by immortalisation through the introduction of the lentiviral vector vBAL39 expressing human telomerase reverse transcriptase (hTERT) (16-19) in a NKNT-3 clonal derivative. The vBAL39 vector carries a CMV promoter controlling the expression of a reverse tetracycline responsive transcriptional activator and Tet responsive element controlling the expression of the hTERT gene (20). The hTERT gene and the lentiviral vector were kindly delivered by R.L. Beijersbergen, Netherlands Cancer Institute and J. Seppen, AMC, The Netherlands (21,22), respectively. Lentiviral transduction was carried out as described (21). After transduction, doxycyclin (1 µg/ml) was added to the medium to activate the tet-on promoter system.

***Table 1**: Four different treatments of NKNT-3 cells and clonal derivatives to investigate reversion of immortalisation and expected composition of the cultures after successful reversion of immortalisation*

Treatment	AxCANCre transduction	Neo^{res} selection	Expected composition of culture
+ +	+	+	100% reverted cells
+ -	+	-	< 100% reverted cells
- +	-	+	Total cell death, control of Neo^{res} selection
- -	-	-	Untreated cells

Reversion of immortalisation

Cre mediated recombination to revert immortalisation of NKNT-3 cells was carried out by transduction of the adenoviral vector AxCANCre (Riken DNA Bank (Tsukuba Life Science Center, Japan). The optimal multiplicity of infection (MOI) of a batch of AxCANCre virus was first determined by infecting NKNT-3 cells with various dilutions of infected culture medium and the relation with the fraction of neomycin resistant (neo^{res}) cells. At day 0 cells were seeded in 6-well culture plates at a density of 200.000 cells/well and from then cultured in CS-C medium without hygromycin B. At day 1 cells were transduced with AxCANCre. A selection for neo^{res} cells was performed by the addition of 500 µg/ml G418 to the medium at day 3 and 7. The applied G418 treatment was tested and found to be lethal to all neomycin sensitive cell

types. In general, the optimal number of neo^{res} cells was found after a 1-hour incubation with 400 µl of culture medium harvested from 293T cells infected with AxCANCre virus and vortexed for 20 seconds prior to transduction.

Set-up hepatic function test

NKNT-3 cells in 6-well primaria plates were tested in triplicate for hepatic functions in four groups as indicated in Table 1 at different days after optional AxCANCre transduction. As a reference, primary hepatocytes were tested at day 2 after seeding. After washing the cells twice using phosphate buffered saline (PBS), 3.5 ml of testmedium consisting of CS-C medium containing 0.5 mM NH_4Cl was added to the cells. One ml of medium was sampled immediately and after eight hours culturing. Then the cells were washed three times using PBS and stored at –20° C for protein determination. In addition, RNA was isolated from NKNT-3 cells at day 12 for reverse transcriptase-polymerase chain reaction (RT-PCR) analyses. As a reference, RNA was isolated from two 0.2-0.5 gram frozen liver samples.

Biochemical assays

Ammonia concentrations were determined by an enzymatic kinetic colorimetric assay using glutamate dehydrogenase and NADPH. Urea concentrations were determined using the blood urea nitrogen test (Sigma Chemical Co). Total protein/well was quantified by spectrometry using Coomassie blue (Bio-Rad). Ammonia elimination and urea production were established by calculating the changes in concentration and corrected for protein content.

Monoxygenase assays

Ethoxyresorufin-O-deethylation (EROD) and pentoxyresorufin-O-deethylase (PROD) activities were measured as described (23), with or without 5□M 3-methylcholantrene induction and with or without 2mM phenobarbital induction for 48-72 hr, respectively. Reaction rates were determined under linear conditions with regard to incubation time. Fluorescence values were converted to pmoles with a calibration curve of resorufin fluorescence and expressed as pmol of resorufin formed per mg of total protein and per min. The 6□-hydroxylation of testosterone was determined by HPLC according to Langouet *et al*. (24).

RT-PCR

Total RNA was isolated from 6-well cultures or liver samples using Trizol (GibcoBRL) and was subsequently treated with DNAse I (Boehringer Mannheim). Real-time RT-PCR reactions to detect cDNAs from the highly expressed genes *SV40T*, *GST*π, *AFP* and 18S rRNA, using SYBR® Green I was performed essentially as described (25). To detect cDNAs from *hTERT*, *Cre recombinase*, *Alpha-1 antitrypsin* (*AAT*), *Albumin* and *Transferrin*, a gene-specific RT

***Table 2**: Primers and conditions used in RT-PCR analysis*

Gene	primers 5' → 3'		Amplicon size (bp)	Real-time RT-PCR conditions		
				[$MgCl_2$] (mM)	Template dilution	Annealing temp. (°C)
18S rRNA	RT S AS	CGAACCTCCGACTTTCGTTT TTCGGAACTGAGGCCATGAT CGAACCTCCGACTTTCGTTCT	151	3	1000 X	68 ↓ 63
AAT	RT S AS	GGGGGATAGACATGGGTATGG ACAGAAGGTCTGCCAGCTTC GATGGTCAGCACAGCCTTAT	181	4		68 ↓ 63
AFP	RT S AS	n.a. TKCCAACAGGAGGCYATGC CCCAAAGCAKCACGAGTTTT	306	5		62 ↓ 55
Albumin	RT S AS	ACTTCCAGAGCTGAAAAGCATGGTC TGAGCAGCTTGGAGAGTACA GTTCAGGACCACGGATAGAT	189	4		68 ↓ 63
Cre recombinase	RT S AS	GCACTAGTCTAATCGCCATCTTCCAGCAGG GTGTTGCCGCGCCATCTGC CACCATTGCCCCTGTTTCACTATC	271	5		68 ↓ 63
GST π	RT S AS	n.a. GCCAGAGCTGGAAGGAGG TTCTGGGACAGCAGGGTC	333	5	10 X	70 ↓ 63
hTERT	RT S AS	TTAATTAATCAGTCCAGGATGGTCTTGAA CGTACTGCGTGCGTCGGTAT GGTGGCACATGAAGCGTAGG	233	3		68 ↓ 63
SV40T	RTS AS	n.a. AAAATGAAGATGGTGGGGAGAAGA CAGGGGGAGGTGTGGGAGGTT	179	4		68 ↓ 63
Transferrin	RTS AS	CCAGACCACACTTGCCCGCTATG GAAGGACCTGCTGTTTAAGG CTCCATCCAAGCTCATGGC	310	2		68 ↓ 63

reaction was performed. First-strand cDNA was generated using a combination of 40 pmol of gene-specific RT-primer (Table 2) and 134 units of Superscript III (Invitrogen) in a total volume of 25 µl at 50 ºC for one hour followed by a 15 min incubation at 70ºC. The resulting gene-specific RT reaction mixtures were column purified using Qiaquick PCR Purification Kit (Qiagen).

Real-time PCR was performed using 1 µl of cDNA and LightCycler FastStart DNA Master SYBR® Green 1 reagent (Roche). Primers and reaction conditions are depicted in Table 2. The thermal cycling profile of the touchdown PCR was as follows: 94 °C for 10 min, followed by 40 cycles of 94 °C for 1 s, primer annealing at high annealing temperature for 7 s with a 0.5 °C/cycle decrease until low annealing temperature, and extension for 40 s at 72 °C. PCR specificity was verified by melting curve analysis and agarose gel electrophoresis. The mRNA quantification was carried out by using the LinRegPCR programme (26). For each sample the mRNA starting level was normalised for the *18S* rRNA starting level. To correct for variations between RT-PCR runs, the mRNA starting levels of liver specific genes of NKNT-3

cells were additionally standardized for average mRNA starting levels of two liver samples that were simultaneously subjected to RT-PCR. The mRNA starting levels of SV40T and Cre recombinase were standardized for the average starting mRNA levels of the NKNT-3 P2 and P28 +- samples.

Statistical analysis

Data were analyzed using SPSS 10.0 software (SPSS Inc.). Results are reported as means ± standard deviation. Levene's test followed by independent samples student T tests were used to compare outcomes between different groups. Spearman's rho (r_s) was used to analyze non-linear correlations. Significance was reached if $P < 0.05$.

Results

Reversion of immortalisation in relation to culturing time

To test the effects of reversion of immortalisation of NKNT-3 cells as a consequence of AxCANCre transduction and selection for neo^{res}, the subconfluent cultures underwent four different treatments (Table 1). As a measure for cell number, the protein content per well was established at different time points after optional treatment in repetitive experiments. Fig. 1A shows the outcomes of a representative experiment using NKNT-3 cells of the second passage after receipt of the cells (P2). The population doubling time of the cells was approximately 40 hours. The neo^{res} selection was effective as the cell number in the -+ culture decreased markedly. When cells were additionally transduced with AxCANCre, a significant fraction of cells became neo^{res} as described previously (9), as can be deduced from the considerable protein content in ++ cultures. The growth of these cultures was inhibited as compared to the -- cultures, however not completely, as can be inferred from the increase in protein content of the ++ cultures. The efficiency of the AxCANCre transduction and/or the Cre/Lox recombination was clearly not complete, since +- cultures continued to grow as the -- cultures until confluency was reached. So, the +- cultures, and to a lesser extent the ++ cultures, consisted out of reverted and immortalised cells.

Upon further passaging of the NKNT-3 culture, however, the cells lost the capacity to become neo^{res} after AxCANCre transduction. This was shown for cells of 28th passage (P28) of which the protein content of ++ and -+ cultures was similarly decreasing in time (Fig. 1B). Duplication of this experiment, starting with a new batch of P2 cells cultured until P28, yielded similar results.

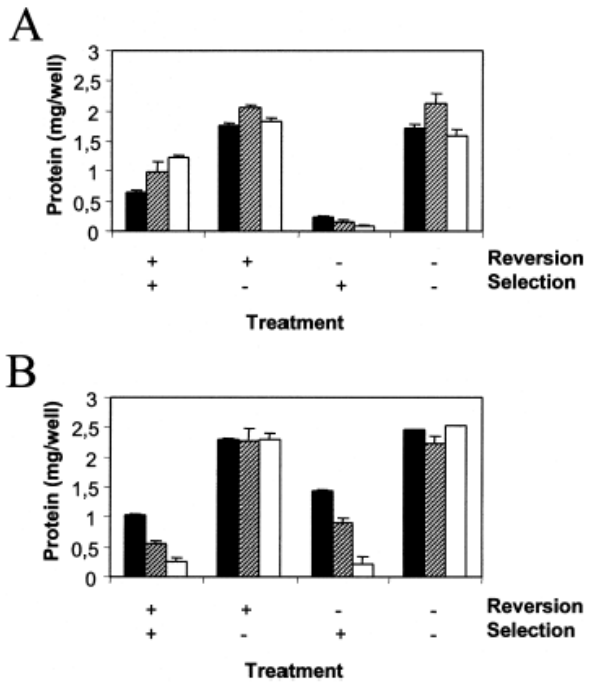

***Figure 1**: Protein concentration of NKNT-3 P2 (A) and P28 (B) cultures in CS-C medium undergoing four different treatments determined at day 8 (black bars) , 11 (striped bars) and 13 (white bars) postoptional AxCANCre transduction (n = 3).*

One reason of loss of capacity to become neo^{res} after AxCANCre transduction might be that the transduction efficiency by AxCANCre was decreased in the P28 cells as compared to P2 cells. However, flow cytometry analysis using an antibody against the coxsackievirus and adenovirus receptor (CAR) showed similar positivity for P2 and P28 cells (data not shown). Moreover, RT-PR showed that the Cre recombinase mRNA levels were similar in P2 +/+, +/- and P28 +/- cultures at 13 days post-optional transduction. The Cre recombinase mRNA level of the P28 ++ culture was threefold lower in comparison to the other AxCANCre transduced cells, however, this was not significant. Therefore, the sensitivity to AxCANCre transduction or the capacity to express Cre recombinase probably had not changed during long-term culturing. To further assess whether Cre/Lox recombination had taken place in the AxCANCre transduced cultures and determine the effect of AxCANCre transduction on the SV40T expression, RT-PCR was carried out at 13 days post-optional transduction (Fig. 2). The SV40T mRNA levels were significantly reduced after AxCANCre transduction and selection for neo^{res} in NKNT-3 populations of P2 and P28 cells. A discrepancy between mRNA levels of cre and SV40T in the P28 ++ and +- cells exists, because both mRNA levels are affected by the efficiency of AxCANCre transduction, however SV40T mRNA levels are further affected by selection for neo^{res}. So, although protein determinations showed that only a minor

fraction of the AxCANCre transduced cells of the P28 cells became neo^{res}, the Cre mediated recombination had taken place.

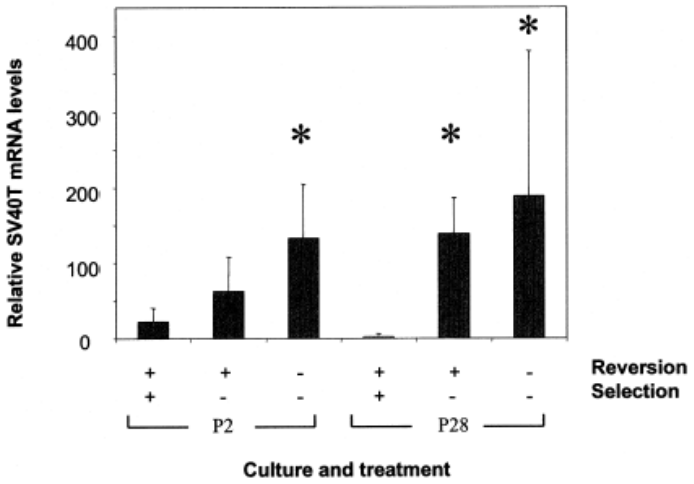

***Figure 2**: Relative SV40T mRNA levels of +/+, +/- and -/- groups of NKNT-3 P2 and P28 cells at day 13 postoptional AxCANCre transduction in CS-C medium determined by using real-time RT-PSR (n = 3). The -/+ treated cells are not included in this analysis due to the insuficient number of cells. * p < 0.05 compared to +/+ group.*

Generation and analysis of NKNT-3 clonal derivatives

Because the NKNT-3 cells acquired another phenotype during expansion, as shown above, and different cell morphologies could be distinguished in the culture, it was hypothesized that the NKNT-3 culture was not a monoclonal cell line, but instead originated from multiple immortalised liver cells. To obtain insight in the heterogeneity of the NKNT-3 culture, six clonal derivatives were generated from P2 cells and then analysed. These cell lines differed considerably in size (Fig. 3) and growth rate. The population doubling time of the three cell lines with small cell sizes, cBAL54, cBAL56 and cBAL57, was 30-40 hours, which was comparable to the initial NKNT-3 culture. However, the population doubling time of the three cell lines with larger cell sizes increased during culturing, starting from 30 hours. Of these slowly growing cell lines, only one, cBAL55, continued to grow with a population doubling time of 80 hours and the other two cell lines stopped proliferating after 15-18 population doublings and became enlarged and pycnotic. Given their necrotic appearance these two cell lines might have entered crisis, due to critical attrition of telomeres (27,28). Therefore, the human telomerase reverse transcriptase (hTERT) expression of the NKNT-3 P2 and P28 cultures and clonal derivatives that continued to grow was determined using real-time RT-PCR. The cell lines that had stopped proliferating could not be assessed, since the number of cells was too

low. The hTERT mRNA levels were similar in the P2 and P28 cultures, but varied by a factor 25 among clonal derivatives that did not enter growth arrest (Fig. 4). To investigate whether a lack of hTERT expression was accountable for the growth arrest observed in two clonal

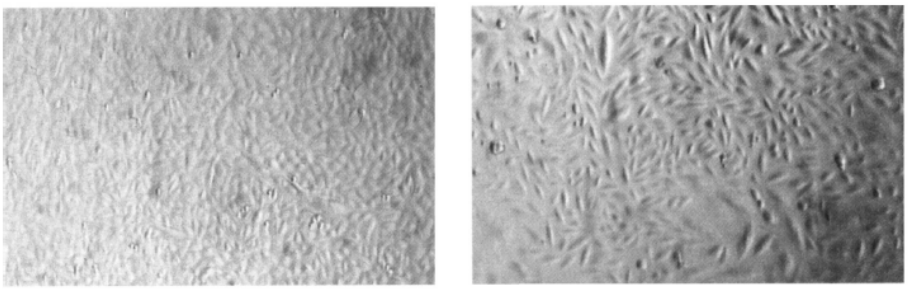

***Figure 3**: Morphology of a fast growing clonal derivative, cBAL56 (left) and slow growing clonal derivative, cBAL55 (right) of NKNT-3 P2 in CS-C medium. Photographs were taken using the same settings*

derivatives, one of these cell lines was transduced with the lentiviral vector vBAL39 expressing hTERT. In a culture of 50.000 cells, ten colonies of fast growing cells appeared after vBAL39 transduction. The cells deriving from these ten colonies were expanded to generate a new cell line designated cBAL58. This cell line had a population doubling time of 35 hours and expressed hTERT at a 15-fold higher level than the P2 culture (Fig. 4). Thus P2 cultures contain cells that are not immortalised and have a finite life-span due to critically shortening of telomeres.

To test whether the clonal derivatives were capable of becoming neo^{res} after AxCANCre transduction, a protein determination was performed on clonal derivatives that had undergone the previously described treatments (Table 1). Only the cell lines derived from slowly growing cell lines, cBAL55 and cBAL58, became neo^{res} after AxCANCre transduction (Fig. 5), in contrast to the fast growing NKNT-3 clonal derivatives (results not shown).

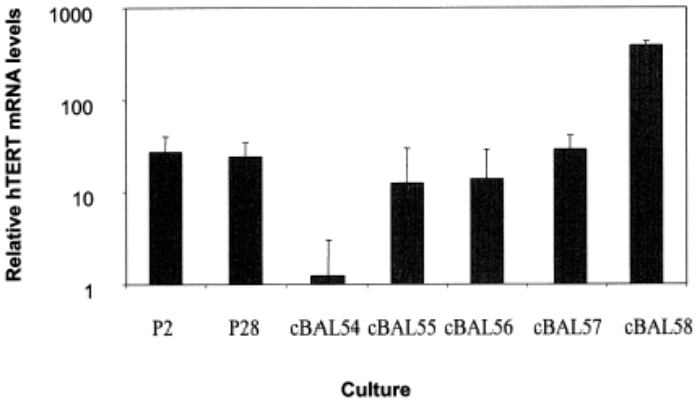

***Figure 4**: Relative hTERT mRNA levels of NKNT-3 P2 and P28 cells and monoclonal derivatives determined by using real-time RT-PCR (n = 3).*

***Table 3**: Characteristics of donors, resected liver samples and primary hepatocyte isolations*

Patient		Sample weight (gram)	Cell isolation	
Age	Gender		Yield (10^6 viable cells / gram)	Viability (%)
28	f	3.7	5.0	83
64	m	9.5	3.4	76
29	f	7.5	5.0	79
68	m	5.0	8.4	81
68	m	44.0	29.6	87
68	m	14.0	4.9	82
70	m	12.5	8.3	80
45	f	6.5	1.7	85

Hepatic functionality of NKNT-3 cells in comparison to human liver cells

To serve as reference material for hepatic functions, primary human hepatocytes were isolated with an average viability of 82% and a yield of 8.3 million viable cells/gram liver from eight liver resection samples as indicated in table 3. These primary hepatocytes were cultured for two days, when functionality was found to be optimal (results not shown), and tested for biochemical parameters similarly as NKNT-3 cells. The cells were tested in three different media: WE, CS-C and ISE-RPMI. The WE medium contains 4%% fetal bovine serum, in contrast to serum-free media CS-C and ISE-RPMI. The WE and ISE-RPMI media are established media for hepatocytes (15,29) and the CS-C medium was used by Kobayashi *et al.* for generating NKNT-3 cells (9). The average ammonia elimination of the primary hepatocytes was the highest in CS-C medium (0.28 $\pm$ 0.15 µmol/h/mg protein) and the average urea production was the highest in ISE-RPMI medium (0.32 $\pm$ 0.14 µmol/h/mg protein (Fig. 6). Two independent groups of NKNT-3 P2 and P28 cells produced on average 0.05 µmol ammonia /h/mg protein, and the urea production was below 0.02 µmol/h/mg protein, irrespective of the treatment the cells had undergone, the culture medium and the period after optional AxCANCre transduction. In addition, 3-dimensional culturing in matrix used for culturing hepatocytes in a bioartificial liver (30) did not yield any improvement of functionality of NKNT-3 cells. To detect hepatic functions of P2 and P28 cells in a more sensitive way, the mRNA levels of different liver-specific genes were compared with *in vivo* levels of two normal liver samples from different patients. The mRNA levels varied less than 50% between the liver samples and also freshly isolated hepatocytes showed similar mRNA levels. The mRNA levels of AAT, albumin and transferrin, as markers for hepatic differentiation, were 4 to 20-fold increased after AxCANCre transduction and neo^{res} selection in the P2 and P28 cells (Fig. 7).

The mRNA levels of the analysed hepatic differentiation markers in the ++ groups of P2 and P28 cells did, however, not exceed 0.1% of detected levels in liver. The mRNA levels of GSTπ and AFP, as markers for hepatic dedifferentiation, were at least 4.5 fold decreased after AxCANCre transduction and neo^{res} selection in the P2 and P28 cells (Fig. 8). The GSTπ mRNA levels were higher in the NKNT-3 cells as compared to liver, except for the P28 ++ culture in which GSTπ expression was undetectable. In contrast, the AFP mRNA levels were lower in NKNT-3 cells as compared to liver. The mRNA levels of both differentiation and dedifferentiation markers were equal between the +- and -- samples of NKNT-3 P2 and P28.

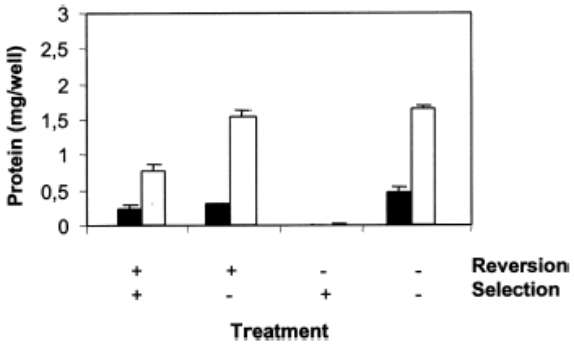

***Figure 5**: Protein concentrations of cBAL55 (black bars) and cBAL58 (white bars) cultrues in CS-C medium that underwent four different treatments determined at day 12 postoptional AxCANCre transduction (n = 3).*

Biotransformation activities were studied in low passage NKNT-3 cells and compared to those in cultured mature human hepatocytes, *i.e.* EROD related to CYP1A1/2, PROD related to CYP 2B6 and 6ß-hydroxylation of testosterone, related to CYP 3A4. NKNT-3 cells metabolized ethoxyresorufin after a 48 hours induction using 3-methylcholanthrene (236$\pm$78 pmol/min./mg *vs.* 432$\pm$167 pmol/min./mg at day 6, respectively). EROD activity was optimum between days 6 and 12 post confluency. However, NKNT-3 cells showed a lower EROD activity compared with that in human hepatocyte cultures (6220$\pm$1180 pmol/min./mg in control *vs.* 138940$\pm$17160 pmol/min./mg in 3-methylcholanthrene treated 6-day-old cultures). In NKNT-3 cells, the 6ß-hydroxylkation of testosterone and PROD activity were neither detectable nor inducible by their known inducers, *i.e.* rifampicine and phenobarbital, respectively.

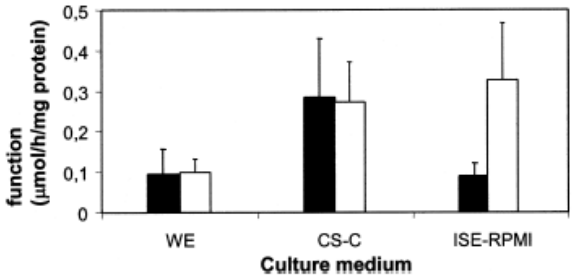

***Figure 6**: Rate of ammonia elimination (black bars) and urea production (white bars) of primary hepatocytes from eight different patients cultured in WE, CS-C, and ISE-RPMI medium and tested at 2 days postseeding (n = 3/sample).*

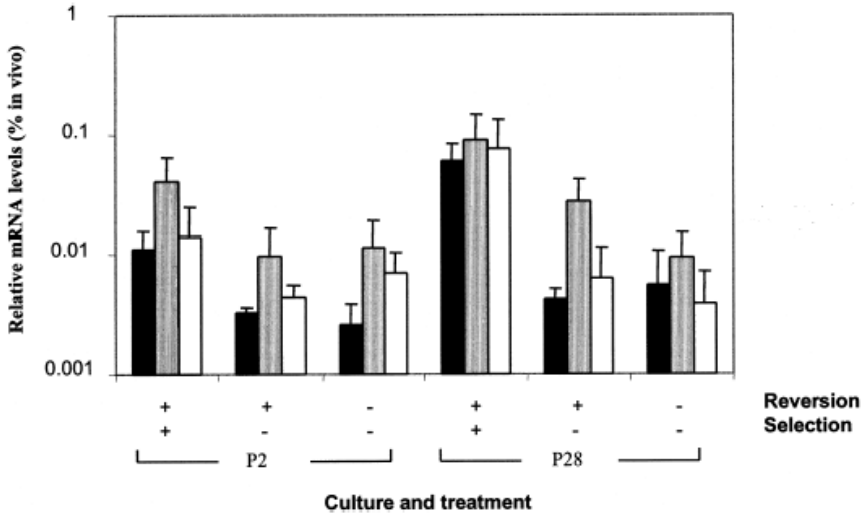

***Figure 7**. Relative AAT (black bars), albumin (gray bars), and transferrin (white bars) mRNA levels of +/+, +/−, and −/− NKNT-3 P2 and P28 cells at day 13 postoptional AxCANCre transduction in CS-C medium determined by using real-time RT-PCR (n = 3). The relative mRNA levels are given as percent of levels determined in two liver samples, normalized for 18S rRNA. The −/+-treated cells are not included in this analysis, due to the insufficient number of cells.*

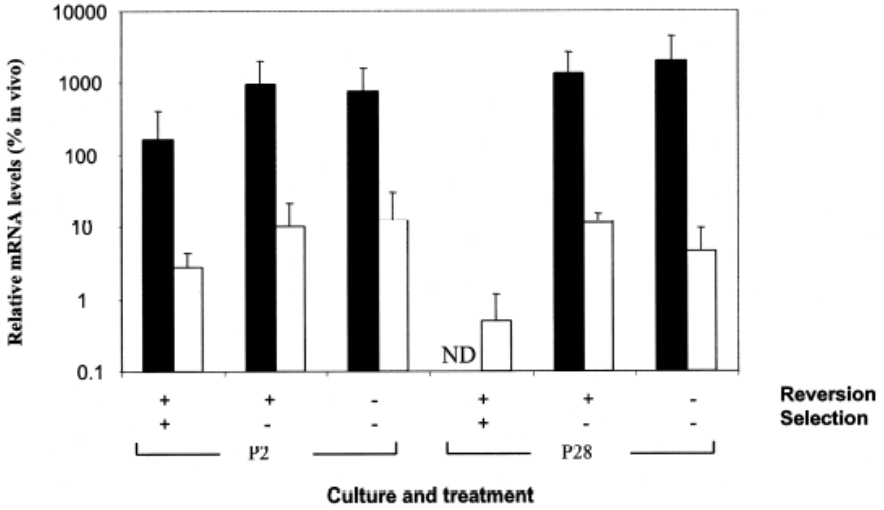

***Figure 8**: Relative GSTπ (black bars) and AFP (white bars) mRNA levels of +/+, +/−, and −/− NKNT-3 P2 and P28 cells at day 13 postoptional AxCANCre transduction in CS-C medium determined by using real-time RT-PCR (n = 3). The relative mRNA levels are given as percent of levels determined in two liver samples, normalized for 18S rRNA. The −/+-treated cells are not included in this analysis, due to the insufficient number of cells. ND: not detectable.*

Discussion

The goal of this study was to assess the NKNT-3 cells for *in vitro* applications. The reproducible loss of the capacity to become neo^{res} after AxCANCre transduction after culturing NKNT-3 for 28 passages, suggests phenotypic instability. The phenotypic changes may be caused by already existing phenotypic variation in the culture or mutations occurring during culturing, both followed by selection for growth advantage. The observation that clonal derivatives isolated from the P2 culture were variable in their capacity to become neo^{res} after AxCANCre transduction, exhibited different population doubling times, and different life-spans, probably related to the critically shortening of telomeres, suggests that the phenotypic variation was already present in the early-passage culture.

Some *in vitro* applications require considerable numbers of hepatocytes, *e.g.* BAL systems are loaded with minimally 10 billion viable hepatocytes (31). To achieve such cell masses, the cell cultures need to undergo 13 population doublings when starting with 1 million cells. Starting with a P2 culture, an increasing proportion of the cells will lack the capacity to become neo^{res} after AXCANCre transduction during expansion. This will impair selection for reversion that is clearly required to accomplish a homogeneous population with increased hepatic differentiation, considering that +- and -- cells display similar hepatic functions. So, for

in vitro applications requiring large cell masses, the NKNT-3 cells and other unstable cell cultures are not suitable, depending on the rate of phenotypic transition.

The reason for selection of cells that loose the capacity to become neo^{res} after AxCANCre transduction is unclear. The continued hygromycin selection pressure during expansion ensured maintenance of the immortalising construct, which was confirmed by the high SV40T expression in P28 -- cells. In addition, AxCANCre transduction led to Cre recombinase expression, and a decrease in SV40T expression, as a result of completed Cre/lox recombination. So, the capacity to be reverted was maintained and only the neo^{res} selection system became defective during long-term culturing. Possibly cells carrying mutations causing defects in the neo^{res} phenotype have been selected. Isolation and sequence analysis of the Neo^R gene of P2 and P28 cultures may reveal selection for a mutation causing defects in the neo^{res} phenotype. We have not fully pursued this yet, since we have no access to the sequence of the immortalising construct and PCR on the Neo^R gene of the immortalising construct failed so far.

For *in vitro* applications requiring only little expansion of cells and not a homogeneous population, the NKNT-3 cells may be applicable. The real-time RT-PCR analyses show that reversion stimulates hepatic differentiation and inhibits dedifferentiation in the P2 and P28 cells. This is reflected, although not significantly, in a negative correlation between mRNA levels of SV40T and liver-specific genes *AAT*, *Albumin* and *Transferrin* ($r_s \leq -0.60$) and, conversely, a positive correlation between mRNA levels of SV40T and dedifferentiation markers GSTπ and AFP ($r_s \geq 0.66$) of the different P2 and P28 cultures. This is also in agreement with the findings of Kobayashi *et al*. using the NKNT3 cells (9) and Cai *et al.* using a conditionally immortalised rat hepatocyte cell line (32), showing increased liver-specific mRNAs after reversion of immortalisation, although not quantitative. However, the hepatic differentiation of the NKNT-3 cells, as judged from urea production, ammonia elimination, monooxygenase activity/inducibility and mRNA levels of hepatocyte-specific genes, is very low in comparison to primary hepatocytes or liver. Thus, the overall effect of reversion of immortalisation on the hepatic functionality is very limited, due to the low initial level of functionality.

The low hepatic differentiation can, at least partly, be explained by the use of SV40T as immortalising factor. SV40T expression commonly leads to malignant transformation as characterized by dedifferentiation, karyotypic instability and in some cases tumorigenicity (33;34). This may have occurred in NKNT-3 cells, since SV40T expression is generally not sufficient to immortalise human cells (35). The NKNT-3 cells were tested negative for tumorigenicity (9), so the stage of malignant transformation may still be limited.

In conclusion, the *in vitro* applicability of NKNT-3 cells is limited, due to its instability and low hepatic functionality, although reversion of immortalisation stimulates differentiation to

some degree. This finding emphasizes that *in vivo* testing of hepatic cell lines is little informative for predicting their value for *in vitro* applications. *In vivo* testing of hepatic functionality of cells includes presenting the optimal tissue microenvironment and paracrine signals and is therefore less stringent than *in vitro* testing. This was actually already suggested by the observation that transplantation of non-reverted and reverted NKNT-3 cells was equally beneficial to the hepatectomized rats (9).

Testing for *in vitro* applications should therefore comprise testing for stability of essential phenotypic characteristics of the cell line and comparison of functionality with primary human hepatocytes.

Acknowledgements

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Chapter 3

In vitro functionality of human fetal liver cells and clonal derivatives under proliferative conditions

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Summary

Mature human hepatocytes are not suitable for large-scale *in vitro* applications that rely on hepatocyte function, due to their limited availability and insufficient proliferation capacity *in vitro*. In contrast, human fetal liver cells (HFLC) can be easily expanded *in vitro*. In this study we evaluated the hepatic function of HFLCs under proliferative conditions, to determine whether HFLCs can replace mature hepatocytes for *in vitro* applications.

HFLCs were isolated from fetal livers of 16 weeks gestation. Hepatic functions of HFLCs were determined in primary culture and after expansion *in vitro*. Clonal derivatives were selected and tested for hepatic functionality. Results were compared to primary mature human hepatocytes *in vitro*.

No differences were observed between primary HFLCs and mature human hepatocytes in albumin production and mRNA levels of various liver specific genes. Ureagenesis was 4.4-fold lower and ammonia elimination was absent in HFLCs. Expanding HFLCs decreased hepatic functions and increased cell streching. In contrast, clonal derivatives had stable functionality and morphology and responded to differentiation stimuli. Although their hepatic functions were higher than in passaged HFLCs, functionality was at least 20 times lower as compared to mature human hepatocytes.

HFLCs cannot replace mature human hepatocytes in *in vitro* applications requiring extensive *in vitro* expansion, since this is associated with decreased hepatic functionality. Selecting functional subpopulations can, at least partly, prevent this. In addition, defining conditions that support hepatic differentiation is necessary to obtain HFLC cultures suitable for *in vitro* hepatic applications.

Key words: hepatocyte, fetal, proliferation, liver function, *in vitro*

Introduction

For *in vitro* applications relying on hepatocytes, like bioartificial liver systems and pharmacological or toxicological assays, mature human hepatocytes are the preferred cell source, because of their excellent function. However, due to their limited availability and insufficient proliferation capacity *in vitro*, mature human hepatocytes are not suitable for large-scale applications. Therefore much effort has been put into the development of an alternative cell source that combines well-differentiated hepatic function with the possibility to expand cell mass *in vitro*. In this respect tumour derived cell lines, originating from hepatoma or hepatoblastoma (15,22) and *in vitro* immortalised cell lines have been investigated (8,18,32). In general, these cell lines exhibit sufficient proliferation capacity, but low hepatic functionality, like urea production from ammonia and cytochrome p450 activity. In addition, in the case of bioartificial liver applications, the use of cells originating from tumours raises questions about the risk of transmigration of tumour cells into the patient (15).

Alternatively, the possibility of using human fetal liver cells (HFLC) for *in vitro* hepatocyte applications has been investigated, because these cells are reported to possess a high proliferation capacity *in vitro* (13). However, their functionality is less well described, which may be due to variations in the fetal age and the culture conditions applied (11,13,21,27). In addition, the functionality of HFLCs is rarely compared to that of primary mature human hepatocytes that are maintained under the same conditions. This comparison is crucial for the evaluation of alternative cell sources, because at this moment the primary mature hepatocytes are considered to be the functional standard for *in vitro* applications.

The level of hepatic functionality of fetal liver cells *in vitro*, is strongly dependent on the proliferation stimulus provided to the cells. When HFLCs are kept under conditions that only mildly stimulate proliferation, hepatic phenotype can be maintained for several weeks (11). HFLCs maintained in primary culture for 10 to 12 months, have been reported to form organoids, containing epithelial structures and connective tissue (11). When fetal liver cell cultures are exposed to proliferation stimulating conditions, their hepatic functionality might decrease. Downregulation of Retinoblastoma protein, which leads to cell-cycle progression, rapidly decreased hepatocyte markers, like albumin and α-fetoprotein (AFP) secretion, in rat fetal liver cells (5). Expansion of fetal liver cells by subculturing may further enhance the dedifferentiation process (13). Serial passaging decreased the number of albumin expressing HFLCs (11) and changed cell morphology from hepatocyte-like shape to flattened and spindle-like (13). This may be explained by a selective advantage of cells optimally adapted to culture conditions and displaying the highest proliferation capacity during culture. An indication in this direction might be given by Lazaro et al., who reported the presence of large and small hepatocytic cells in HFLC cultures and observed that smaller cells became more

evident during prolonged culture (11). A second possibility is that HFLCs undergo a transition during *in vitro* culturing, analogous to the epithelial-mesenchymal transition (EMT) described for fetal and neonatal rat hepatocytes in culture (17,30). Downregulation of hepatocyte nuclear factor (HNF) 1α and 4 characterizes the dedifferentiation process occurring during EMT (30). Although the underlying mechanism of the changes in proliferative HFLC cultures are unclear, the instability of the HFLC cultures implicates that selection is necessary to obtain a homogenous cell population or that HFLCs cannot be extensively expanded *in vitro*, pending an improvement of differentiating culture conditions.

In the present study we examined the possibility of using HFLCs under proliferative conditions for *in vitro* applications as an alternative for mature human hepatocytes. The hepatic function of HFLCs was determined before and during expansion *in vitro* and compared to the function of mature human hepatocytes under the same culture conditions. Furthermore we investigated whether stable cultures could be obtained by selecting clonal derivatives and whether hepatic function could be stabilized or induced.

Materials and Methods

Cell isolation and culture.

Human fetal livers were obtained from elective abortions. Gestational age was determined by ultrasonic measurement of the diameter of the skull and ranged from 14 to 18 weeks. The use of this tissue was approved by the Medical Ethical Committee of the Academic Medical Center and was contingent on informed consent. We isolated HFLCs on three independent occasions; in each case four fetal livers were pooled. HFLCs were isolated and cultured by methods adapted from the protocol described by Malhi et al. (13). Fetal livers were digested with 0.03% collagenase P (Roche) in Hank's balanced salt solution (BioWhittaker) for 30 minutes at 37°C. Dissociated cells were pelleted at 160 g for 5 minutes at 4°C and washed two times with DMEM culture medium (Dulbecco's modified Eagle's medium, BioWhittaker) containing 10% heat-inactivated fetal bovine serum (HI-FBS, BioWhittaker), 2 mM L-glutamine (BioWhittaker), 1 μM dexamethason (Sigma), 10 μg/mL insulin, 5.5 μg/mL transferrin, 6.7 ng/mL selenium-X (ITS mix, Life Technology), 100 U/mL penicillin, 100 μg/mL streptomycin (penicillin/streptomycin mix, BioWhittaker)). HFLCs were seeded in DMEM culture medium at a density of approximately $3 \cdot 10^5$ cells/cm^2 in Primaria 6-well plates (BD Falcon). To obtain clonal derivatives, a limiting dilution assay was performed after the second passage of the HFLCs. To support the cells during this single cell state, cells were seeded in Primaria 96-well plates and DMEM culture medium was enriched with 50% culture medium conditioned for 24 h by HFLCs that were passaged twice. This regime was terminated and

DMEM culture medium, as described above, was used when cells reached confluence and were transferred from the 96-well plates. Clonal derivatives were selected based on morphology. Cells with a fibroblast-like morphology, defined as spindle-shaped cells were excluded; cells with an epithelial morphology, defined as cubic cells, were further analysed.

Near-confluent cultures were split at 1:4 ratios. Cells were detached by 5 min incubation with 0.25 % trypsin / 0.03% EDTA (BioWhittaker). The number of population doublings (PD) was calculated as PD = log (N_f/N_i) / log 2, in which N_f is the final number of cells harvested and N_i is the number of initially seeded cells. No corrections were made for cells that did not re-attach after passaging, since their proportion was negligible. PD time (T_{PD}) was calculated over the period in which PD number progressed linear with culture time.

Mature primary human hepatocytes were isolated from seven 2-10 gram non-tumour liver tissue from patients undergoing partial hepatectomy, because of secondary carcinoma. The procedure was approved by the Medical Ethical Committee of the Academic Medical Center and was contingent on informed consent. Hepatocyte isolation method was adapted from the protocol described by Seglen (26). Briefly, liver tissue was flushed through the veins with ice-cold Ringers glucose solution (NPBI) containing 10 U/mL heparin (Leo Pharma), until it was free of blood. Then 100 mL of calcium-free buffer (137 mM NaCl (Merck), 2.7 mM KCl (Merck), 0.5 mM EGTA (Boehringer Mannheim), 10 mM HEPES (Roche), 10 mM glucose (Merck), 0.7 mM Na_2HPO_4 (Merck), 100 IU/mL penicillin-G (Yamanouchi), 40 mg/L gentamycin (BioWhittaker), 100 mg/L vancomycin (Eli Lilly), 2 mg/L flucanozol (Diflucan, Pfizer Inc.), pH 7.5) at 37°C was perfused through the liver tissue for 10 minutes. To dissociate the cells, the tissue was then perfused with 100 mL digestion buffer (137 mM NaCl, 2.7 mM KCl, 5 mM$CaCl_2$ (Merck), 10 mM HEPES, 10 mM glucose, 0.7 mM Na_2HPO_4, 0.4 g/L collagenase P (Roche), 100 IU/mL penicillin-G, 40 mg/L gentamycin, 100 mg/L vancomycin, 2 mg/L flucanozol, pH 7.5) at 37°C for 15 minutes. Digested liver was sliced into small pieces and filtrated through surgical gauze. Dissociated liver cells were pelleted at 50 g for 3 minutes at 4°C and washed twice with WE culture medium (William's E medium with 4% HI-FBS, 2 mM L-glutamine, 1 µM dexamethason, 20 mU/mL insulin (Novo Nordisk), 2 mM ornithine (Sigma-Aldrich), 100 U/mL penicillin, 100 µg/mL streptomycin). The viability and yield was determined by trypan blue exclusion test. Hepatocytes were seeded in WE culture medium at a density of 10^5 cells/cm^2 in Primaria 6-well plates. All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Hepatic function tests

Hepatic function was tested two days after seeding mature hepatocytes and HFLCs or at confluence of passaged HFLCs and clonal derivatives in 6-well plates. Culture medium was replaced by 2.5 mL of test medium (WE culture medium with 0.5 mM NH_4Cl) after washing

the cells twice with phosphate buffered saline (PBS, NBPI International). Medium samples were taken after 0 and 8 (mature hepatocytes) or 72 (HFLCs and clonal derivatives) hours of incubation. The cells were then washed twice with PBS and stored at -20°C for protein determination. All experiments were performed in triplicate.

In the differentiation experiments, confluent cultures of HFLCs and clonal derivatives were incubated for four days in WE culture medium containing 1% dimethylsulfoxide (DMSO, Sigma), 10 mM nicotinamide (NA, Sigma), 10 ng/mL oncostatin M (OSM, Research diagnostics, inc.), 5 mM sodiumbutyrate (Sigma), 1 µM all-trans retinoic acid (RA, Sigma), 10 ng/mL hepatocyte growth factor (HGF, BD Biosciences) or 10 ng/mL fibroblast growth factor 4 (FGF-4, Research diagnostics, inc.). After this incubation, cells were tested in the presence of these compounds as described.

PCNA staining

Freshly isolated HFLCs were seeded into 8-wells culture-slides (BD Falcon) in DMEM medium as described. Two or six days after seeding, the cells were washed twice with PBS and fixated by a 10 minute incubation with ice-cold methanol-aceton-water mixture (2:2:1). Slides were stored in 70% ethanol at 4°C. To detect proliferating cell nuclear antigen (PCNA), slides were washed three times 5 minutes in PBS, then incubated for 30 minutes with Teng-T (10 mM TRIS-HCl, 5 mM EDTA, 150 mM NaCl, 0.25% gelatine, 0.05% Tween-20, pH 8.0) and for 2 hours with Teng-T containing 200 ng/mL anti-PCNA antibody (Santa Cruz, sc-56). Slides were washed three times 5 minutes in PBS, incubated for 1 hour in PBS containing 2 µg/mL goat-anti-mouse IgG antibody-Alexa594 conjugated (Molecular Probes, A11005), washed three times 5 minutes in PBS and mounted in Vectashield mounting medium containing diamidinophenylindoldiacetate (DAPI, Vector Laboratories). Photographs were made with a Leica DM RA2 microscope using a Leica DC350F digital camera and FW4000 software. Instrument settings were kept constant for all photographs.

RT-PCR

RNA was isolated from HFLCs and clonal derivatives that were cultured for 72 hours in WE culture medium. As a reference for hepatocyte specific genes, RNA was isolated from two 0.2-0.5 gram snap frozen liver samples. RNA was isolated using TRIzol (Boehringer Mannheim). And as a reference for cholangiocyte specific genes, RNA from two cholangiocyte cell lines, Mz-ChA-1 and Sk-ChA-1 was kindly delivered by M. Lie-a-Ling and C.T. Bakker (Academic Medical Center, Amsterdam). First strand cDNA was generated from 500 ng of total RNA using 20 pmol of gene-specific RT primer for the mRNA of albumin, α-1-antitrypsin (AAT), transferrin, AFP, π class glutathione S transferase (GST π), hepatocyte nuclear factor 4 (HNF4), HNF1α, Cytochrome P450-3A4 (Cyp3A4), Cyp3A7, cytokeratin 7

(CK7) and CK19 in combination with 5 pmol of RT primer for 18S ribosomal RNA and 134 units Superscript III (Invitrogen). Real-time reverse transcription PCR (RT-PCR) using SYBR green I (Roche) was performed as described previously (6). The sequences of the RT and PCR primers and PCR conditions are given in Table 1.

Starting levels of mRNA were calculated by analyzing linear regression on the Log (fluorescence) per cycle number data using LinRegPCR software (19). Starting levels of mRNA calculated for albumin, AAT, transferrin, AFP, GSTπ, HNF4, HNF1α, Cyp3A4, Cyp3A7, CK7 and CK19 were normalised for the starting levels of 18S ribosomal RNA. Normalised mRNA levels are expressed as a percentage of the mean starting levels of the two liver samples normalised for 18S ribosomal RNA starting levels.

Biochemical assays

Ammonia concentration was determined by an enzymatic kinetic colorimetric assay using glutamate dehydrogenase and NADPH, performed in a P800 Roche Diagnostics analyzer. Urea concentration was determined using a blood urea nitrogen test according to the instructions of the manufacturer (Sigma Chemical Co). Total protein/well was quantified by spectrometry using Coomassie blue (Bio-Rad). Albumin concentration was determined by enzyme linked immunosorbent assay using cross-absorbed goat-anti-human albumin antibodies (Bethyl).

Statistics.

Results are reported as mean ± standard deviations of at least three independent experiments. Student's *t* test was performed to compare outcomes. Results were considered to be significant if $p < 0.05$. Messenger RNA levels are reported as the mean of duplicate experiments, no statistical analysis was performed on these results.

***Table 1**: Primer sequences and real-time RT-PCR conditions.*

Gene	primers 5' → 3'		Amplicon size (bp)	Real-time RT-PCR conditions		
				[$MgCl_2$] (mM)	Template dilution	Annealing temp. (°C)
18S rRNA	RT S AS	CGAACCTCCGACTTTCGTTT TTCGGAACTGAGGCCATGAT CGAACCTCCGACTTTCGTTCT	151	3	1000 X	68 ↓ 63
AAT	RT S AS	GGGGGATAGACATGGGTATGG ACAGAAGGTCTGCCAGCTTC GATGGTCAGCACAGCCTTAT	181	4		68 ↓ 63
AFP	RT S AS	CGTTTTGTCTTCTCTTCCCC TKCCAACAGGAGGCYATGC CCCAAAGCAKCACGAGTTTT	306	5		62 ↓ 55
Albumin	RT S AS	ACTTCCAGAGCTGAAAAGCATGGTC TGAGCAGCTTGGAGAGTACA GTTCAGGACCACGGATAGAT	189	4		68 ↓ 63
CK 7	RT S AS	AGGACCCGCACTGCTGGAGAAG CTGGGAAGCATGGGGACGACCTC CAATGCCACCGCCACTGCTACTGC	404	2		68 ↓ 63
CK 19	RT S AS	TCAGTAACCTCGGACCTGCT GCAGATCGAAGGCCTGAA TGAACCAGGCTTCAGCATC	208	2		68 ↓ 63
GST π	RT S AS	AGCAGGTCCAGCAG GTTG GCCAGAGCTGGAAGGAGG TTCTGGGACAGCAGGGTC	333	5	10 X	70 ↓ 63
Transferrin	RT S AS	CCAGACCACACTTGCCCGCTATG GAAGGACCTGCTGTTTAAGG CTCCATCCAAGCTCATGGC	310	2		68 ↓ 63
HNF1α	RT S AS	AGCCCCTGTGCCTGTGATGG GGAGGCGGCCCACCAGAAAG TCCGCCCTATTGCACTCCTC	436	3		68 ↓ 63
HNF4α	RT S AS	CACTCCAACCCCGCCCCTC CCGGGTGTCCATACGCATCCT CAGGTTGTCAATCTTGGCC	321	3		68 ↓ 63
Cyp3A4	RT S AS	CCCGTGAGAAGCAGAGGA AGCTTAGGAGGACTTCTTCAACC AGCCAAATCTACCTCCTCACACT	313	3		68 ↓ 63
Cyp3A7	RT S AS	AGCCAAATCTACTTCCCCAGCAC ATTACGCTTTGGAGGACTTCTTCT CGTCTTCATTTCAGGGTTCTATTT	182	3		68 ↓ 63

RT = primer used in reverse transcriptase reaction, S = sense primer, AS = antisense primer, 68 ↓ 63 = 'touchdown' annealing temperature, starting at 68°C, decreasing 0.5°C/cycle until 63°C is reached, using 63°C as annealing temperature until the end of the program.

Results

Morphology of HFLCs in vitro.

HFLCs were isolated from fetal livers of 14 to 18 weeks gestation and taken into culture. We chose to test HFLCs of 16 weeks gestational age, because these cells still exhibit a high proliferation capacity and a relatively long life span. The HFLCs performed 57.6 ± 10.2 PDs in culture with a T_{PD} of about two days before entering a state of terminal growth arrest. HFLCs isolated at 23 weeks gestational age had a reduced life span of 28 PDs and a T_{PD} of 3 days (n=1).

Primary HFLCs initially consisted mainly of clusters of cells with hepatocyte morphology (Fig.1a), but the percentage of cells with fibroblast-like morphology rapidly increased in the first week and the hepatocyte-like cell clusters became surrounded by cells with a fibroblast-like morphology (Fig. 1c). The addition of HGF and/or EGF did not affect cell growth rate neither did it inhibit the overgrowth by fibroblast like cells (data not shown). Although not all cells in primary cultures were PCNA positive, cells of all morphologies stained positive for PCNA in cultures of both two and six days (Fig 1b,d) and were therefore actively dividing. Passaging of primary HFLCs was associated with rapid loss of the dominant hepatocyte morphology. However, early passages still showed a variety of morphologies, ranging from cells with a cubic, epithelial-like appearance (marked with C in Fig. 2a), to stretched, fibroblast-like cells (marked with S in Fig. 2a). When subculturing was pursued, HFLCs eventually became homogenous populations of fibroblast-like cells.

Hepatic functionality of HFLCs in vitro.

Cells were tested for hepatic function and expression patterns at different time points during culturing and compared to primary mature human hepatocytes under the same culture conditions. Primary HFLCs, tested two days after isolation, produced similar amounts of albumin compared to primary mature human hepatocytes (Table 2). Urea production rate of primary HFLCs was 4.4-fold lower compared to primary mature human hepatocytes ($p < 0.05$, Table 2). In HFLCs both these functions rapidly decreased after serial passaging. After eight PDs, completed after four passages, urea and albumin production rate were decreased 11- and 480-fold, respectively ($p < 0.05$). Primary mature human hepatocytes showed an ammonia elimination rate of 95.2 ± 60.7 $nmol \cdot h^{-1} \cdot mg\ protein^{-1}$. No ammonia elimination was detected for primary HFLCs.

To determine whether serial passaging also reduced mRNA levels of hepatic genes, we performed RT-PCR analyses (Table 2). The mRNA levels of genes associated with hepatic maturation *i.e.* albumin, transferrin, AAT, HNF1α and HNF4 in primary HFLCs were

***Table 2**: Functional and transcriptional characteristic of primary and passaged HFLCs and 6 clonal derivatives.*

Function / Cells	Hepatic function		Mature hepatic marker (mRNA levels %)						Immature hepatic marker (mRNA levels %)			Cholangiocyte marker (mRNA levels %)	
	Albumin production ($ng \cdot h^{-1} \cdot mg^{-1}$ protein)	Urea production ($nmol \cdot h^{-1} \cdot mg^{-1}$ protein)	Albu-min	Trans-ferrin	AAT	HNF 1α	HNF 4	Cyp 3A4	GSTπ	AFP	Cyp 3A7	CK7	CK19
Primary MHH	37.7 ± 7.8	91.5 ± 33.7	130	245	121	*ND*	*ND*	*ND*	535	*ND*	*ND*	*ND*	*ND*
Primary HFLC	47.1 ± 15.9	20.5 ± 7.3 [a]	28.6	42.9	180.5	178.6	195.6	0.19	1924	6818	578	*ND*	*ND*
HFLC after 8PDs	0.08 ± 0.09 [b]	0.6 ± 0.5 [b]	1.00	0.84	0.16	24.4	50.9	0.05	296	21	20	*ND*	*ND*
cBAL08	0.27 ± 0.37	4.0 ± 1.6 [c]	0.02	0.28	0.22	24.9	21.1	0.00	1081	28	0	353	67
cBAL09	0.03	0.9	0.02	0.11	0.62	*ND*	*ND*	*ND*	808	80	*ND*	310	81
cBAL20	0.42 ± 0.19	2.6 ± 3.0	0.01	0.04	0.01	12.4	*ND*	0.00	249	21	19	1689	58
cBAL21	0.19 ± 0.32	0.7 ± 0.7	0.01	0.16	0.02	68.9	12.2	0.00	432	120	0	17	179
cBAL24	0.02	8.6	0.02	0.12	0.02	*ND*	*ND*	*ND*	852	61	*ND*	*ND*	*ND*
cBAL29	0.48 ± 0.14 [c]	5.5 ± 1.1 [c]	0.02	0.12	0.01	24.4	34.8	0.00	1059	137	0	*ND*	248
Mz-ChA-1	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	6963	25858
Sk-ChA-1	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	3282	1823

The mRNA levels are expressed as percentage of mRNA levels detected in mature human liver in vivo.
[a] $p < 0.05$ compared to primary MHH
[b] $p < 0.05$ compared to primary HFLC
[c] $p < 0.05$ compared to HFLC PD8
MHH = mature human hepatocytes, HFLC = human fetal liver cells. ND = not determined

***Table 3**: Induction of albumin and urea production of cBAL08, cBAL29 and HFLCs after 12 PDs by differentiating additives in culture medium and the effects of these additives on the mRNA levels of indicated markers of cBAL08*

Function / Additive	Hepatic function (fold induction compared to basic)						mRNA levels cBAL08 (fold induction compared to basic)				
	Albumin production			Urea production			Mature hepatic marker			Immature hepatic marker	
	cBAL08	cBAL29	HFLC PD12	cBAL08	cBAL29	HFLC PD12	Albumin	Trans-ferrin	AAT	GSTπ	AFP
Basic	1.0 ± 0.3	1.0 ± 0.5	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 0.3	1.0 ± 0.6	1.0	1.0	1.0	1.0	1.0
1% DMSO	1.3 ± 1.6	1.9 ± 1.3	1.0 ± 0.6	1.4 ± 0.6	2.0 ± 1.2	0.4 ± 0.3	0.7	1.2	0.7	0.6	0.4
10 mM NA	0.6 ± 0.5	0.9 ± 0.5	1.0 ± 0.3	0.7 ± 0.3	1.8 ± 0.4 [a]	0.7 ± 0.3	1.9	1.9	1.8	0.9	0.8
10 ng/mL OSM	1.7 ± 1.0	1.3 ± 1.2	0.9 ± 0.2	0.8 ± 0.7	1.5 ± 0.5	0.7 ± 0.2	0.6	0.9	0.4	0.6	0.2
5 mM Butyrate	1.5 ± 1.5	1.1 ± 1.2	1.1 ± 0.9	2.2 ± 0.3 [a]	2.2 ± 0.5 [a]	1.7 ± 0.5	1.1	0.2	0.9	0.7	0.2
1 µM RA	2.0 ± 2.4	1.1 ± 0.3	0.9 ± 0.6	1.5 ± 0.6	2.1 ± 1.4	0.8 ± 0.3	1.2	11.3	0.0	0.5	0.4
10 ng/mL HGF	0.6 ± 0.8	1.6 ± 1.0	1.0 ± 0.1	0.7 ± 0.3	1.1 ± 0.4	0.7 ± 0.6	1.1	1.4	1.3	1.0	1.0
10 ng/mL FGF-4	1.3 ± 1.4	1.3 ± 1.0	0.6 ± 0.3	0.5 ± 0.2 [a]	1.4 ± 0.4	0.4 ± 0.2 [a]	0.6	1.2	1.2	1.0	0.7

[a] *$p < 0.05$ compared to basic medium*

between 29% and 196% of mature *in vivo* levels. After eight PDs the mRNA levels of albumin, transferrin and AAT were reduced to less than 1.% of the mature *in vivo* levels, whereas HNF1α and HNF4 mRNA levels were reduced 7.4- and 38-fold to 24% and 51%, respectively. Cyp3A4 mRNA levels were more than 500-fold lower in primary HFLCs, when compared to mature *in vivo* levels and decreased 3.7-fold after 8 PDs. Genes associated with immature hepatocytes, *i.e*. GSTπ and AFP, showed a similar pattern: primary HFLCs displayed GSTπ and AFP mRNA levels 19- and 68-fold higher than mature *in vivo* expression levels, respectively, which reduced to 293% and 21% after eight PDs. MessengerRNA of Cyp3A7, the predominant fetal cytochrome P450 isoform (27), was only detectable in one of the two mature liver samples and Cyp3A7 mRNA levels of primary HFLCs were 5.8-fold higher than in this mature liver sample. Again, mRNA levels decreased, almost 30-fold after 8 PDs. Thus, the mRNA levels of diverse hepatic genes decreased after serial passaging.

Morphology and functionality of clonal derivatives.

To investigate the phenotypic instability of the HFLC cultures, clonal derivatives were acquired by limiting dilution. Clones with an epithelial-like morphology were selected for further investigation, whereas clones with a fibroblast-like morphology were excluded. A selection of representative clones is presented in Fig. 2b-e. The morphology of the selected clones was stable until the cells reached terminal growth arrest. Eight clonal derivatives were studied in detail for their growth characteristics. As shown in Fig. 3, all clones displayed a longer T_{PD} and a reduced life span as compared to the original HFLC cultures, except for cBAL08, which reached a maximal number of PDs of 61.

We performed hepatic function tests in three independent experiments on the six clonal derivatives that could undergo at least 30 PDs. No statistical analysis was performed on the results of cBAL09 and cBAL24, because these cultures could not be expanded in an adequate amount for three replicate experiments. Table 2 shows the urea and albumin producing capacity of the six clones tested after at least 30 PDs. The clones differed in their functionality. Functionality seemed to be preserved in some clonal derivatives when compared to the HFLC PD8 cultures. Two clones, cBAL08 and cBAL29, produced more urea than the HFLC PD8 cultures ($p < 0.05$) and cBAL29 produced more albumin ($p < 0.05$). In cBAL08 and cBAL29, urea production rates were stable with increasing PD number (Fig. 4). Urea production of cBAL08 and cBAL29 was between 4 and 6% of the mature primary hepatocytes and albumin production of cBAL29 was 1.3%. So, functionality of the clonal derivatives was still insufficient.

At the level of gene expression, no clear preservation of function was observed in clonal derivatives, even though differences are observed between clonal derivatives (Table 2). For example, cBAL20 was the only cell line in which Cyp3A7 was detected.

To determine if the clonal derivatives exhibited a cholangiocyte phenotype, the mRNA levels of two cholangiocyte markers, CK7 and CK19 were determined. The levels of these markers in cBAL08, cBAL09, cBAL21 and cBAL29 were comparable to mature liver *in vivo* and at least nine times lower than in the cholangiocyte cell lines Mz-ChA-1 and Sk-ChA-1. Only the CK7 mRNA level of cBAL20 was comparable to that of the cholangiocytes.

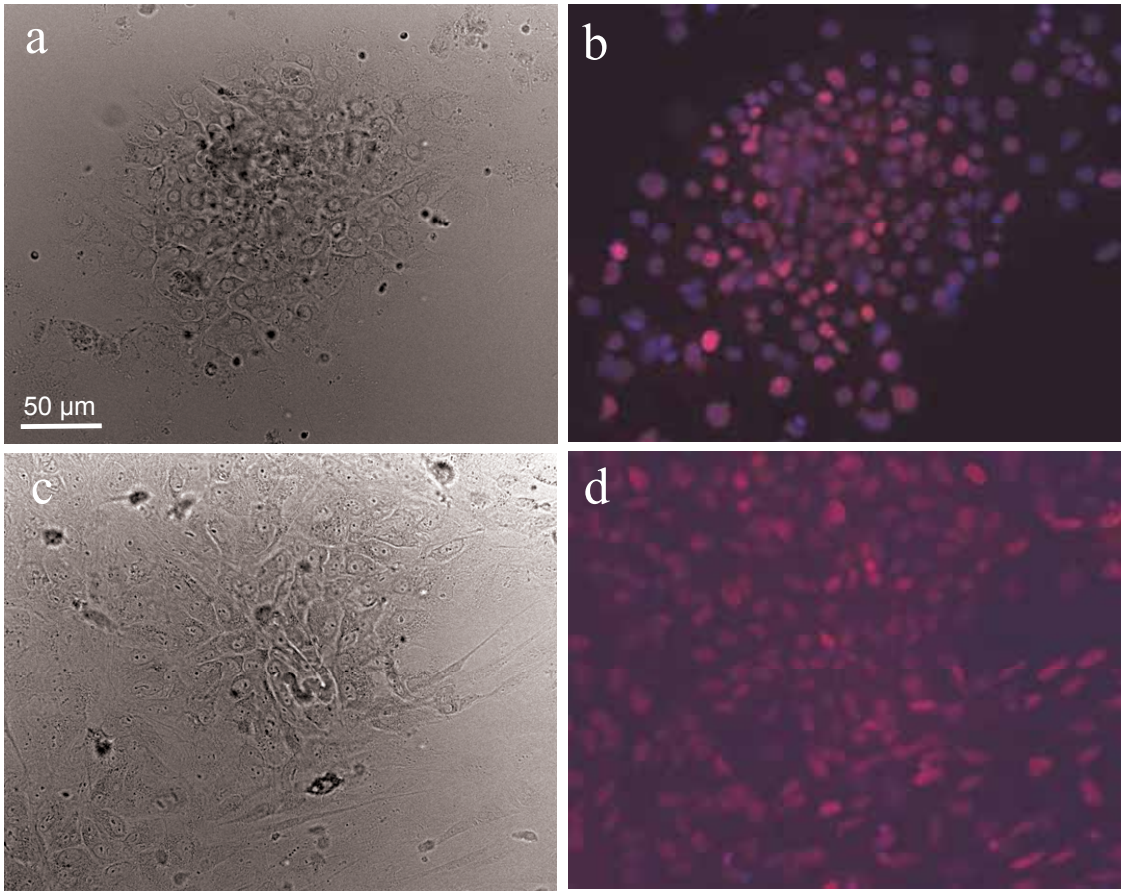

***Figure 1**: Brightfield (a, c) and fluorescence (b, d) micrographs, in which the nuclei are stained with DAPI (blue) and PCNA is visualised with Alexa 594 (red). (a) Cell cluster with hepatocyte morphology in primary HFLC culture, two days after seeding and (b) same cluster stained for PCNA. (c) Cell cluster with hepatocyte morphology in primary HFLC culture, six days after seeding and (d) same cluster stained for PCNA.*

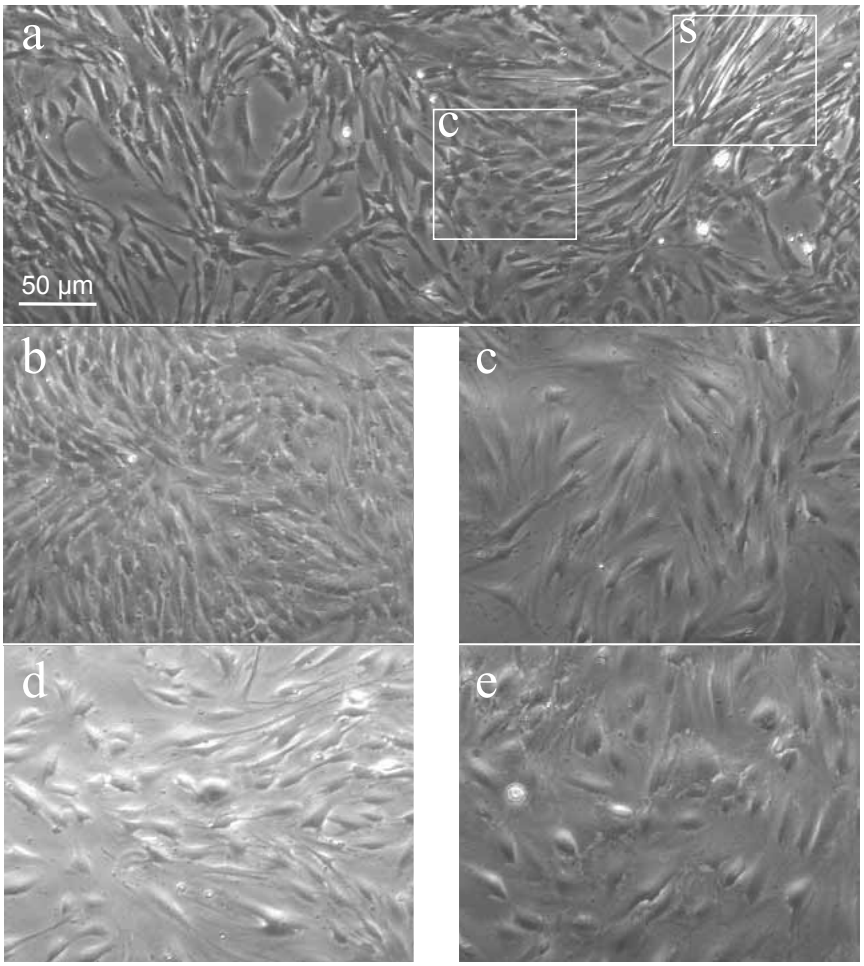

***Figure 2**: Microscopy of passaged HFLC cultures after 4 PD (a) and four representative clonal derivatives: cBAL08 (b), cBAL20 (c), cBAL21 (d) and cBAL24 (e).*

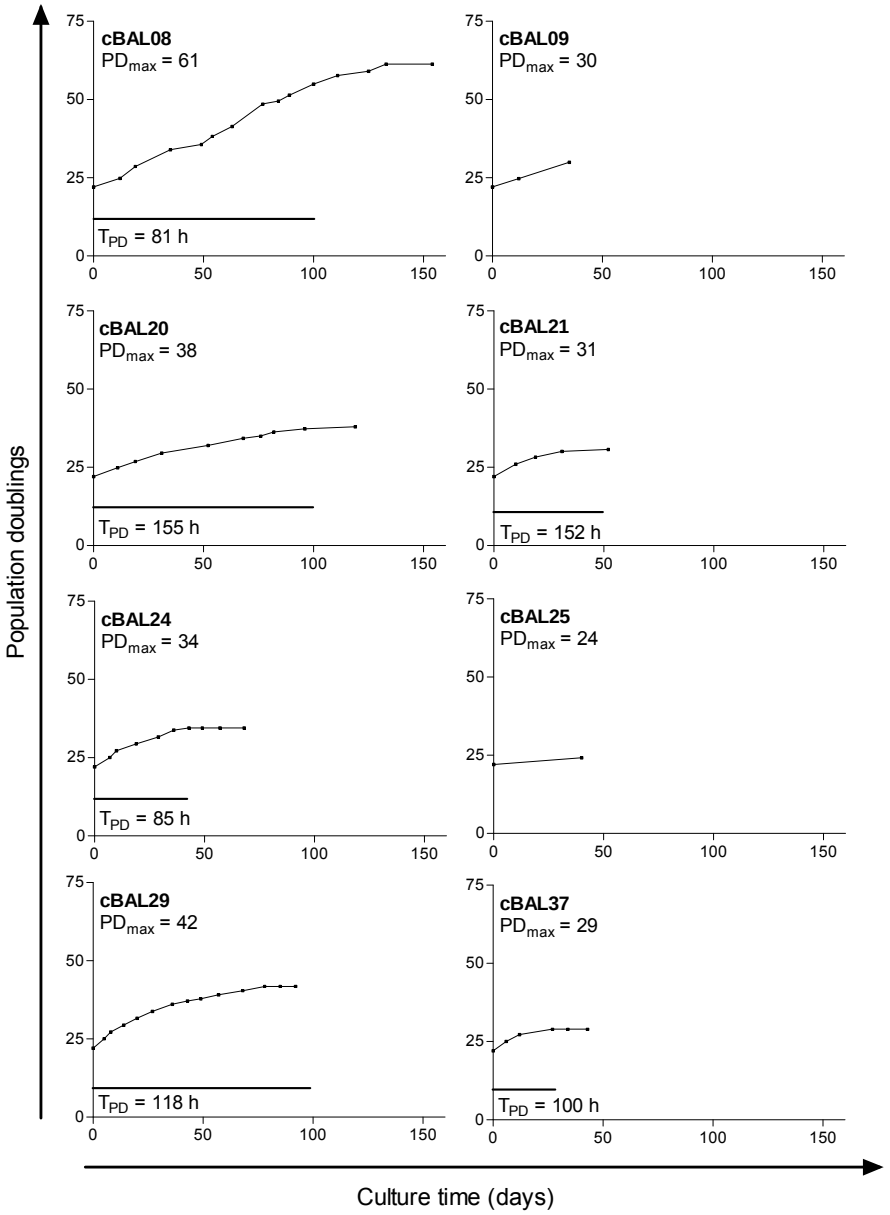

***Figure 3**: Growth curves of 8 clonal derivatives. The black bars indicate the period over which the PD time (T_{PD}) is calculated.*

Differentiation experiments.

To enhance hepatic functionality, cells were incubated with agents that are reported to induce or preserve the hepatic phenotype *in vitro* of fetal liver cells, mature hepatocytes, bone marrow cells or embryonic stem cells. For these studies we used HFLCs after 12 PDs and the two clonal derivatives with the highest hepatic function, *i.e*. cBAL08 and cBAL29, after they performed at least 20 PDs. Urea synthesis increased 1.8-fold by 10 mM nicotinamid in cBAL29 and 2.2-fold by 5 mM sodiumbutyrate in cBAL08 and cBAL29 ($p < 0.05$), however no ammonia elimination was detected. In cBAL08 10 ng/mL FGF-4 lowered urea synthesis two-fold ($p < 0.05$). Other compounds showed no significant induction of function. The clonal derivatives seemed to be more responsive to differentiation stimuli than the HFLC cultures, however this is not statistically significant. No significant effects were detected on albumin synthesis.

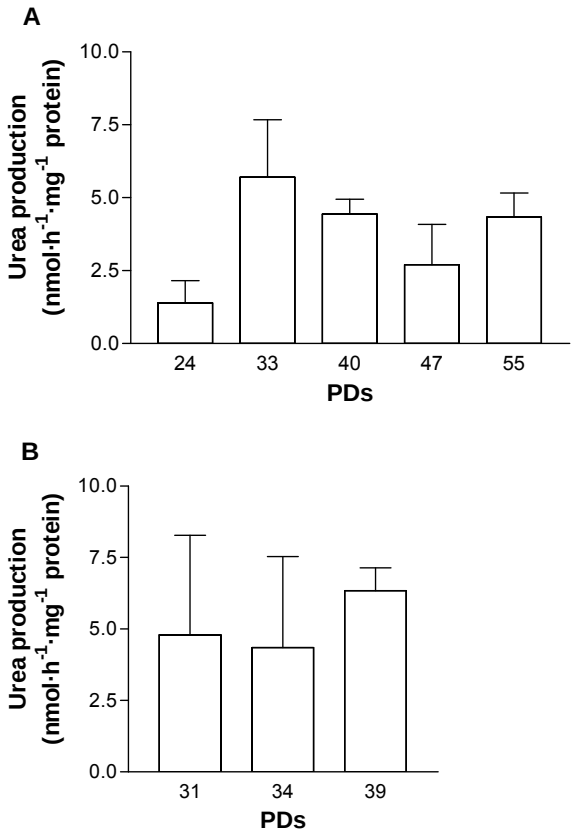

***Figure 4**: Urea production rate of cBAL08 (A) and cBAL29 (B) during long-term culture.*

At the level of gene transcription, 10 mM nicotinamid increased albumin, transferrin and AAT mRNA levels of cBAL08 almost two-fold without affecting GSTπ and AFP mRNA levels. Treatment of cBAL08 with 1 μM retinoic acid upregulated transferrin mRNA levels more than 11 times, while it almost abolished AAT mRNA levels. This effect was confirmed in cBAL29 in which retinoic acid caused an 8.7-fold increase of transferrin mRNA level (data not shown).

Discussion

For a reliable evaluation of an alternative cell source for *in vitro* hepatic function, comparison with mature human hepatocytes under the same conditions is of vital importance. To our knowledge, this comparison has never been reported for HFLCs. We show that the functionality of primary HFLC cultures is comparable to that of primary mature human hepatocytes for albumin production and the mRNA levels of albumin, transferrin, AAT, HNF1α and HNF4. More complex functions, like expression of cytochrome p450 and urea production from ammonia, were lower in HFLCs when compared to mature hepatocytes.

Ureagenesis is 4.4-fold lower in HFLCs compared to mature human hepatocytes and ammonia elimination was not detected in HFLCs. So, HFLCs isolated at 16 weeks gestation do not exhibit a functional urea cycle *in vitro*. This is probably a reflection of the *in vivo* functionality of fetal liver. In subjects with inborn urea cycle deficiencies, the ammonia accumulation starts shortly after birth (12). This indicates that the ammonia removal *in utero* can take place through the placenta and only after birth the liver becomes the site of action. Cyp3A4 mRNA levels are more than 500-fold lower and Cyp3A7 mRNA levels are 5.8-fold higher in primary HFLCs when compared to mature liver. This reciprocal relation between Cyp3A4 and Cyp3A7 and its shift during liver development is a well known phenomenon (27).

Primary HFLCs can be easily expanded *in vitro*. In this study, HFLCs were expanded in culture medium based on DMEM enriched with 10% HI-FBS to support proliferation, as described earlier (13,31). Culture medium was enriched with a mixture of insulin, transferrin and selenium as adapted from Lazaro et al. (11) and dexamethasone was added to maintain *in vitro* hepatic differentiation (7,9,20). However, these supplements could not prevent the progressive loss of hepatic morphology and functionality during eight PDs. This loss of function is consistent with the loss of function in progenitor liver epithelial cells during eight passages reported previously (13). The addition of HGF and/or EGF did not affect cell growth of the epithelial cells nor the fibroblast like cells, possible because of the high serum percentage of the medium, which contains many growth factors.

Changes in morphology and functionality of the HFLC culture result, at least partly, from the heterogeneity of the isolated cell population. Part of the cells with hepatocyte morphology present in the primary cultures is PCNA positive, thus proliferating, but are rapidly outnumbered by cells with fibroblast-like morphology. After passaging HFLCs, the dominance of hepatocyte morphology is lost. This might be a result of the trypsin treatment, which is known to damage cell-cell connections and cell-extracellular matrix connections (16). Extracellular matrix provides hepatocytes with signals important for the maintenance of differentiation status (1). Another explanation for the loss of the hepatocyte morphology can be that the cells undergo EMT, as described for fetal rat hepatocytes (30). However, the high levels of HNF1α and HNF4 mRNA in passaged HFLCs and the clonal derivatives contradict the observed low HNF1α and HNF4 mRNA levels in EMT (30). Finally, the epithelial cells in the HFLC cultures may have been overgrown by fibroblast-like cells. When the cells with an epithelial morphology were clonally expanded, their population was stable until terminal growth arrest occurred, but they grew slower than the HFLCs and the life span of most of the clones was shorter. Thus, selecting the optimal cell type can partly stabilise the phenotype and prevent overgrowth by fibroblast-like cells.

Although heterogeneity of the HFLC population is involved in the functional and morphological instability of the cultures, dedifferentiation of the cells, due to applied culture conditions, cannot be excluded. Optimizing culture conditions may inhibit or even reverse this process. Therefore we selected compounds that are reported to induce or preserve the *in vitro* hepatic phenotype of fetal liver cells, mature hepatocytes, bone marrow cells or embryonic stem cells (3,14,23,25) and tested their effects on cBAL08, cBAL29 and HFLCs after 12 PDs. Induction of urea production rate was possible in cell line cBAL29 and cBAL08, but was limited with a maximum of a 2-fold induction by addition of butyrate. No induction of both urea and albumin production was observed by the addition of DMSO, oncostatin M, retinoic acid, HGF and FGF-4. None of the studies that described the differentiating effects of compounds we selected, used HFLCs, which may explain the discrepancies found. Furthermore, the continued proliferation under the applied culture conditions may not support drastic hepatic differentiation *in vitro* as *in vitro* proliferation and differentiation seem to be mutually exclusive.

Our limiting dilution assay did not select for hepatic functionality, but only for morphology. This may also explain the limited functionality of the clonal derivatives. However, the low mRNA levels of cholangiocyte markers in most of the cell lines that were selected, combined with HNF1α and 4 levels that are comparable to mature human hepatocytes indicate that the clonal derivatives are indeed early hepatocytes rather than cholangiocytes.

Many studies aim at finding cellular markers that can select the most functional cell types from primary HFLC isolates (10,28,29). However, because of the limited discrimination capacity of available markers, resulting cell populations are still heterogeneous and

consequently instable when expanded. Therefore we decided to investigate clonally expanded cell lines selected for an epithelial morphology.

Primary HFLCs have a number of functions at levels comparable to mature hepatocytes, but whether HFLCs can be used as an alternative for mature human hepatocytes for *in vitro* applications, depends on the requirements of the application. The differences in substrate specificity of the mature Cyp3A4 and the fetal Cyp3A7 can hamper the use of HFLCs as an alternative for mature human hepatocytes for *in vitro* pharmacological and toxicological studies (33). For bioartificial liver applications, HFLCs might be an attractive cell source: HFLCs have considerable synthetic functions and do exhibit a detoxification system, although of immature consistency. However, HFLCs do not eliminate ammonia under the applied culture conditions. Since hyperammonemia is one of the causes of complications in acute liver failure (4), HFLCs will only be useful for bioartificial liver applications if culture conditions are optimized to support a functional urea cycle. Alternatively, ammonia removal might be achieved by up-regulation of glutamine synthetase (2) or other urea cycle enzymes or by combining a bioartificial liver system with haemodialysis (24). The possibilities mentioned above, are applicable for primary HFLCs only. *In vitro* expansion of HFLCs rapidly decreased all hepatic functions and should be avoided pending improved culture conditions. This eliminates the advantage of HFLCs over mature human hepatocytes for large-scale applications.

In conclusion, we have shown that the synthetic functions of primary HFLCs are comparable to those of mature human hepatocytes. However the functionality rapidly reduced upon expansion of these cells *in vitro*, at least partly due to overgrowth of fibroblast-like cells. In addition, the HFLCs do not eliminate ammonia and display an immature CYP profile. These findings impair future *in vitro* applications of HFLCs. However, selection of a subpopulation of cells results in stable cell cultures and can partly overcome the loss of functionality. Therefore the next steps to exploit HFLCs for *in vitro* applications should comprise the selection of a functional subpopulation of HFLCs combined with cell culture conditions that stimulate or stabilize hepatic differentiation *in vitro*.

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C h a p t e r 4

Novel immortalised human fetal liver cell line, cBAL111, has the potential to fully differentiate into functional hepatocytes

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Abstract

A clonal cell line that combines both stable hepatic function and proliferation capacity is desirable for *in vitro* applications that depend on hepatic function, such as pharmacological or toxicological assays and bioartificial liver systems. Therefore, cell clones were derived from human fetal liver cells and immortalised by lentiviral over-expression of telomerase reverse transcriptase. The resulting cell line, cBAL111, showed albumin and urea production, mRNA levels of hepatocyte-specific genes similar to the parental cells prior to immortalisation, but did not show any evidence of growth in soft agar. Cell line cBAL111 expressed albumin, cytokeratin 18 and 19 and showed high glutathione S transferase π mRNA levels. In contrast to hepatic cell lines NKNT-3 and HepG2, all hepatic functions and markers were expressed in cBAL111, although there was considerable variation in their levels compared with primary mature hepatocytes. When transplanted in the spleen of immunodeficient mice, cBAL111 engrafted into the liver and partly differentiated into hepatocytes showing expression of carbamoylphosphate synthetase without any signs of cell fusion. In conclusion, this novel immortalised fetal liver cell line has the potential to differentiate into mature hepatocytes to be used for *in vitro* hepatocyte applications.

Key words: Telomerase, Immortalisation, Differentiation, HepG2, NKNT-3

Introduction

Most pharmacological or toxicological assays and bioartificial liver support systems require fully differentiated hepatocytes. The availability of mature human hepatocytes is variable and the numbers low, because they are usually isolated from donor livers not suitable for transplantation. In addition these cells hardly proliferate *in vitro* [2;8]. Since mature human hepatocytes cannot be used for large-scale applications, there is a pressing need for a cell line that combines highly differentiated hepatic functions while maintaining adequate proliferation capacity.

Several cell lines derived from human liver tumours, such as the hepatoma cell line HepG2 [17], as well as *in vitro* immortalised cell lines, like the NKNT-3 cell line, have been investigated [7;9]. In general, these cell lines proliferate adequately, but the levels of hepatocyte-specific functions (*e.g.* urea production from ammonia and cytochrome p450 detoxification activity) remain disappointedly low.

In tumour-derived cell lines, the mutations leading to immortalisation are largely unknown. In an attempt to control the immortalisation process and therefore prevent at least part of the dedifferentiation process, several immortalised cell lines have been developed. However, although certain genetic modifications in immortalised cell lines are known, spontaneous mutations contributing to the immortalisation cannot be excluded.

For successful *in vitro* immortalisation, overexpression of cell cycle stimulating genes is generally required. Due to the low proliferation capacity of mature hepatocytes, strong stimulation of cell cycle progression is necessary for immortalisation. In the majority of *in vitro* immortalisations of primary human liver cells, the gene encoding Simian Virus 40 Large T antigen (SV40T), an inhibitor of the cell cycle inhibitors p53 and the Retinoblastoma protein, has been used [9;11;14;19]. In addition, overexpression of Cyclin D1, which stimulates cell cycle progression, and dominant negative mutants of p53 have also led to successful immortalisation [29]. In some immortalised cell lines, proliferation was combined with stabilisation of the telomeres [16]. Critically short telomeres induce a terminal state of growth arrest called crisis [3]. Overexpression of the reverse transcriptase of telomerase, hTERT, stabilizes telomere length, thereby avoiding cellular crisis. As a general principle immortalisation by overexpression of hTERT only, minimises the reduction in functionality [27].

In contrast to mature human hepatocytes, fetal human hepatocytes have the ability to proliferate *in vitro* [10;27] and thereby can be immortalised without cell cycle stimulation. In addition, telomere stabilisation in itself can immortalise these cells [27]. Wege *et al.* showed that the immortalisation of fetal human hepatocytes did not affect their differentiation potential, however, the functionality of the immortalised cells was not compared with mature

human hepatocytes [27]. Such comparison is essential to establish the suitability of these cells for hepatocyte applications *in vitro*. Furthermore phenotypical stability of these cells may be low, since these were not of clonal origin. In our previous report we demonstrated that primary human fetal liver cells (HFLCs) in culture exhibit albumin production rates and hepatocyte specific mRNA levels comparable to those of primary mature human hepatocytes *in vitro* [4]. However, after eight population doublings most of these functions were decreased to less than 1% of the corresponding function of primary human hepatocytes *in vitro*. This functional loss can be, at least partly, attributed to the presence of non-parenchymal cells in the cell preparation, which eventually outnumber the functional hepatocytes. Therefore, selection of functional cell clones is necessary if HFLCs are extensively expanded *in vitro*.

In a previous study we already isolated HFLCs and selected specific clones based on their morphology and growth potential [4]. This study combines the telomerase based immortalisation technique with the selection of functional HFLCs to obtain new hepatic cell lines. The resulting immortalised cell line was tested for hepatic functionality *in vitro* and *in vivo*. The *in vitro* functionality was compared with other well-known hepatic cell lines, more specifically the conditionally immortalised NKNT-3 [9] and the tumour derived HepG2 cells [17]. To test whether the resulted novel immortalised cell line had the ability to differentiate into functional hepatocytes, the cell line was transplanted into the spleen of immunodeficient mice.

Materials and Methods

Cell isolation and culture

Human fetal livers were obtained from elective abortions. Gestational age was determined by ultrasonic measurement of the diameter of the skull and ranged from 14 to 18 weeks. The use of this tissue was approved by the Medical Ethical Committee of the Academic Medical Center, Amsterdam, the Netherlands, subject to informed patient consent. We isolated HFLCs on three independent occasions; in each case four fetal livers were pooled. Cells were isolated as described previously [4]. HFLCs were seeded in DMEM culture medium (Dulbecco's modified Eagle's medium, BioWhittaker) containing 10% heat-inactivated fetal bovine serum (HI-FBS, BioWhittaker), 2 mM L-glutamine (BioWhittaker), 1 µM dexamethason (Sigma), 10 µg/mL insulin, 5.5 µg/mL transferrin, 6.7 ng/mL selenium-X (ITS mix, Life Technology), 100 U/mL penicillin, 100 µg/mL streptomycin (penicillin/streptomycin mix, BioWhittaker) at a density of approximately $3*10^5$ cells/cm^2 in Primaria 6-well plates (BD Falcon). Clonal derivatives were obtained by limiting dilution. The selection procedure used

and the functionality of the clonal derivatives are described elsewhere [4]. Near-confluent cultures were detached by 5 min incubation with 0.25 % trypsin / 0.03% EDTA (BioWhittaker) and split at 1:4 ratios. The number of population doublings (PD) was calculated as PD = log (N_f/N_i) / log 2, in which N_f is the final number of cells harvested and N_i is the number of cells initially seeded. No corrections were made for cells that did not re-attach after passaging, since their proportion was negligible. The period in which PD number progressed linear with culture time was used to calculate the PD time (T_{PD}).

Mature primary human hepatocytes were isolated from seven patients undergoing partial hepatectomy, because of metastatic carcinoma. The tumour free liver tissue used in each case for the hepatocyte isolation ranged between 2 to 10 grams. The procedure was approved by the Medical Ethical Committee of the Academic Medical Center subject to informed patient consent. The hepatocyte isolation method was adapted from the protocol described by Seglen [22] as previously described [4].

NKNT-3 cells were kindly donated by Prof. I. Fox, University of Nebraska, USA. The NKNT-3 cells were cultured on Primaria 6-well culture plates (BD Falcon) and in 75 cm2 culture flasks using CS-C complete serum free medium (Cell Systems Corporation) with 0.2 mg/ml hygromycin B (Invitrogen) and 1 U/ml penicillin/streptomycin (BioWhittaker). Cultures were passaged with a split ratio of 1:5 according to instructions for CS-C medium. Cre-mediated recombination to revert immortalisation of NKNT-3 cells [9] was carried out by transduction of the adenoviral vector AxCANCre (Riken DNA Bank (Tsukuba Life Science Center, Japan) as described previously [7]. We analysed both reverted, hence transduced with AxCANCre and selected with G418, and unreverted *i.e.* untreated cells. HepG2 cells were obtained from ATCC (HB-8065) and cultured in Primaria tissue culture flasks in DMEM culture medium as described above for HFLCs.

All cultures were maintained at 37ºC in a humidified atmosphere (95% air, 5% CO2) and the medium was changed every 2-3 days.

Introduction of hTERT and Green Fluorescent Protein genes

The cDNA of the human telomerase reverse transcriptase (hTERT) gene, kindly provided by R.L. Beijersbergen, Netherlands Cancer Institute, the Netherlands, was introduced in the cells by lentiviral transduction. The lentiviral vector backbone was described as LTRCMVR2 by Marcusic et al. [13] and was produced as previously described [23]. In brief, HEK293T cells were transiently transfected by calcium phosphate precipitation with a third generation lentiviral vector system. Virus containing supernatant was collected at 24 and 48 hours following transfection, filtered through 0.45 µm Millipore filters, concentrated by centrifugation and added to the culture medium of the HFLCs. The lentiviral vector contained a cytomegalovirus promoter controlling the expression of a reverse tetracycline (Tet)

responsive transcriptional activator and a Tet responsive element controlling the expression of the hTERT gene. In this system hTERT transcription was increased by adding 1 µg/mL doxycyclin to the medium. The hTERT cDNA was introduced in three independent HFLC cultures and in five clonal derivatives, *i.e.* cBAL08, cBAL09, cBAL20, cBAL21 and cBAL29. Cells were passaged twice before integration of the lentiviral vector was confirmed by PCR using genomic DNA of transduced cells as template.

In the transplantation experiment cBAL111 cells were marked with Green Fluorescent Protein (GFP) by transduction using lentiviral construct pRRLcpptPGKGFPpreSsin [23] carrying the GFP gene under control of a phosphoglycerate kinase promoter. By fluorescence-activated cell sorting it was demonstrated that $\geq$ 95% of the transduced cells were GFP positive. There were at least five passages between transduction with GFP and transplantation of the cells.

Hepatocyte function tests

Hepatocyte function tests were performed at confluence in 6-well plates. After washing the cells twice with phosphate buffered saline (PBS, NBPI International) culture medium was replaced by 2.5 mL of test medium (William's E medium with 4% HI-FBS, 2 mM L-glutamine, 1 µM dexamethason, 20 mU/mL insulin (Novo Nordisk), 2 mM ornithine (Sigma-Aldrich), 100 U/mL penicillin, 100 µg/mL streptomycin, 0.5 mM NH_4Cl). Medium samples were taken after 0 and 72 hours of incubation. The cells were then washed twice with PBS and stored at -20°C for protein determination. All experiments were performed in triplicate.

Biochemical assays

Urea concentrations were determined using the blood urea nitrogen test (Sigma Chemical Co). Albumin concentrations were determined via enzyme linked immunosorbent assays using cross-absorbed goat-anti-human albumin antibodies (Bethyl). Total protein/well was quantified by spectrometry using Coomassie blue (Bio-Rad). Production rates were established by calculating the changes in concentration during time and corrected for protein content.

RT-PCR

RNA was isolated from the cell lines by using TRIzol (Boehringer Mannheim). As a reference, human liver samples were included in the analyses. First strand cDNA was generated from 500 ng of total RNA using 20 pmol of gene-specific RT primers specific for the mRNA of Albumin, α-1-Antitrypsin (AAT), Transferrin, Alpha-fetoprotein (AFP), π class Glutathione S transferase (GST π) and hTERT in combination with 5 pmol of RT primer for 18S ribosomal RNA and 134 units Superscript III (Invitrogen). Real-time reverse transcription

PCR (RT-PCR) using SYBR green I (Roche) was performed as described previously [6]. The sequences of the RT and PCR primers and PCR conditions are given in Table 1.

Starting levels of mRNA, except for hTERT, were calculated by analyzing linear regression on the Log (fluorescence) per cycle number data using LinRegPCR software [21]. Starting levels of hTERT mRNA were calculated by standard curve analysis, using serial dilutions of hTERT containing plasmid ranging from 10^2 to 10^9 copies/reaction and LightCycler software (Roche). The mRNA starting levels of Albumin, AAT, Transferrin, AFP, GST π, and hTERT were normalised for the starting levels of 18S ribosomal RNA. Normalised mRNA levels, except for hTERT mRNA levels, are expressed as a percentage of the mean mRNA starting levels of the two liver samples normalised for 18S ribosomal RNA starting levels.

Immunocytochemistry

For the detection of glutamine synthetase (GS), cBAL111 cells were cultured on 8-wells culture-slides (BD Falcon) for two days. Then the cells were washed twice with PBS and fixed by 10 minutes incubation with ice-cold methanol-aceton-water mixture (2:2:1). Cells were incubated with 70% ethanol for 5 minutes, washed with PBS and subsequently incubated overnight with monoclonal GS antibody (Transduction Laboratories, Lexington, KY, G45020) diluted 1:1000. Antibody binding was visualized with the indirect unlabelled antibody peroxidase anti-peroxidase (PAP) method [24].

For the detection of albumin, cytokeratin (CK) 18 and 19, cBAL111 cells were seeded on Immunoslides (ICN, Aurora, Ohio, USA) and cultured for two and 15 days. Cells were washed once in PBS with 0.1% Tween-20 and fixated in 4% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were washed as before and incubated with a blocking buffer (3% BSA, 0,2% Fishgelatin (Sigma), 2%FCS) for one hour at room temperature. After a further washing step, the cells were incubated with the primary antibody for one hour at room temperature. As primary antibodies we used anti-albumin antibody (Sigma), mouse-anti-human CK18 (sc-6259, Santa Cruz) and mouse-anti-human CK19 (Santa Cruz) for detection of albumin, CK 18 and 19, respectively. Cells were washed again as before and incubated with 28 µg/mL Cy2 conjugated goat-anti-mouse IgG and 1 µg/mL tetramethylrhodamine isothiocyanate (TRITC) conjugated phalloidin for 1 hour in a humidified chamber at room temperature. Forty minutes before the end of this incubation period, 20 ng/mL Diamidinophenylindoldiacetate (DAPI) was added. Cells were washed again as before and embedded in Polymount (Polyscience, Washington, USA) and covered with a coverslip. Slides were analysed using an Axiovert 200 fluorescence microscope.

***Table 1**: Primers and conditions used in RT-PCR analysis. Sequences of the primers used in Reverse Transcriptase (RT) reaction or during real-time PCR reaction (PCR) and the conditions used in the real-time PCR reactions.*

Gene	primers 5' → 3'		Amplicon size (bp)	Real-time RT-PCR conditions		
				[$MgCl_2$] (mM)	Template dilution	Annealing temp. (°C)
18S rRNA	RT S AS	CGAACCTCCGACTTTCGTTT TTCGGAACTGAGGCCATGAT CGAACCTCCGACTTTCGTTCT	151	3	1000 X	68 ↓ 63
AAT	RT S AS	GGGGGATAGACATGGGTATGG ACAGAAGGTCTGCCAGCTTC GATGGTCAGCACAGCCTTAT	181	4		68 ↓ 63
AFP	RT S AS	CGTTTTGTCTTCTCTTCCCC TKCCAACAGGAGGCYATGC CCCAAAGCAKCACGAGTTTT	306	5		62 ↓ 55
Albumin	RT S AS	ACTTCCAGAGCTGAAAAGCATGGTC TGAGCAGCTTGGAGAGTACA GTTCAGGACCACGGATAGAT	189	4		68 ↓ 63
GST π	RT S AS	AGCAGGTCCAGCAG GTTG GCCAGAGCTGGAAGGAGG TTCTGGGACAGCAGGGTC	333	5	10 X	70 ↓ 63
Transferrin	RT S AS	CCAGACCACACTTGCCCGCTATG GAAGGACCTGCTGTTTAAGG CTCCATCCAAGCTCATGGC	310	2		68 ↓ 63

Soft Agar assay

Cells were added to 0.35% low-melting-temperature agarose (Seaplaque) containing DMEM culture medium as described above and transferred at a density of 5000 cells/well to 6-well plates previously lined with 0.5% agar DMEM culture medium. After 15 days, the colonies were stained with 0.005% Crystal violet and counted.

Transplantation

The cBAL111 cells overexpressing GFP were transplanted into 6 week old Rag2-/-γc-/- mice [28]. The mice were anesthetized with an intraperitoneal injection of FFM mixture (2.5 mg Fluanisone/0.105 mg Fentanyl citrate/0.625 mg Midazolam HCl/kg in H_2O, 7 mL/kg). One million GFP-marked cBAL111 cells suspended in 100 µL HBSS were injected into the inferior tip of the spleen as described [20]. Nine or 34 days after transplantation, liver and spleen were harvested after *in vivo* fixation. For *in vivo* fixation, mice were anesthetized as described above and 25 mL PBS was flushed trough the circulation, followed by 25 mL 2% paraformaldehyde (PFA) in PBS. Subsequently, liver and spleen were harvested and cut into pieces of approximately 0.2 cm^3. The tissue pieces were incubated in 4% PFA in PBS for four hours, followed by an overnight incubation in 30% sucrose solution. Tissues were snap frozen in liquid nitrogen and stored at –80°C.

Immunohistochemistry

Cryosections were 6 µm thick and were mounted on poly-L-lysine coated slides. Sections were incubated in Teng-T (10 mM Tris, 5 mM EDTA, 0.15 M NaCl, 0.25% gelatin and 0.05% Tween-20, pH 8.0) for 30 minutes before incubation with primary antibodies. Human mitochondria were visualized with a mouse-anti-human mitochondria antibody (Chemicon International) in a 150-fold dilution; vimentin was visualized with a mouse-anti vimentin antibody, clone 9 (Boehringer Mannheim) in a 1000-fold dilution; carbamoylphosphate synthetase (CPS) was visualized with rabbit-anti-CPS antibody in a 1500-fold dilution and glutamine synthetase (GS) was visualized with monoclonal GS antibody (Transduction Laboratories) in a 500-fold dilution. As a secondary antibody Alexa594 conjugated goat-anti mouse IgG (Molecular Probes) was used in a 1000-fold dilution for the detection of vimentin and human mitochondria and in a 250-fold dilution for detection of GS. CPS antibodies were detected with Alexa594 conjugated goat-anti rabbit IgG (Molecular Probes) in a 250-fold dilution. Slides were mounted in Vectashield containing 1 µg/mL 4,6-diaminidino-2-phenylindole (DAPI) to counterstain DNA.

FISH analysis

Fluorescent in situ hybridization (FISH) analysis was performed on 6 µm sections of paraffin embedded liver tissue as described before [15]. Briefly, sections were treated to remove paraffin and the sections were denatured in 70% formamide for 2.5 minutes. 150 ng Biotin 11-dUTP labeled human genomic DNA, 200 ng digoxigenin 11-dUTP labeled murine genomic DNA and 5 µg salmon sperm DNA were denatured together and hybridized overnight at 37 ºC with the sections. After washing the slides, human DNA was visualized with FITC anti-avidin followed by biotinylated anti-avidin antibodies (Vector laboratories), whilst mouse genomic DNA was visualized with sheep rhodamine anti-digoxigenin followed by Texas red anti-sheep antibodies (Vector laboratories). Slides were mounted in Vectashield (Vector laboratories) containing 1 µg/mL DAPI to counterstain DNA.

Results

Human TERT introduction in HFLCs and clonal derivatives

The maximum number of PDs between the HFLC preparations and clonal derivatives were different. Expression of hTERT was detected in some of the clonal derivatives analysed, however, all HFLCs and clonal derivatives eventually entered a state of terminal growth arrest (Table 2).

After lentiviral introduction of the hTERT gene, the presence of the hTERT cDNA was confirmed in HFLCs and clonal derivatives by PCR on genomic DNA (results not shown). However, the HFLC cultures did not overcome the terminal growth arrest after the introduction of the hTERT gene; only one of the HFLC cultures' lifespan was extended by 30%. In addition, only one of the clonal derivatives was able to overcome the terminal growth arrest after the introduction of hTERT. This clone, cBAL08, previously showed the longest life span of 61 PDs and a relatively high endogenous hTERT expression (Table 2). Because the transduced cell line was capable of more than 120 PDs, which is twice the life span of the parental cell line cBAL08, and still did not show any sign of growth arrest, we considered this cell line to be immortalised and named it cBAL111. The hTERT mRNA levels of cBAL111 were $1x10^5$-fold higher as compared to its parental cell line cBAL08 (Table 2).

***Table 2**. The life span and the hTERT mRNA levels of three different HFLC isolates, clonal derivatives and cBAL111. Life span is indicated as the maximum number of population doublings (PDs).*

Cell source	Maximal PDs	hTERT mRNA copies / 18S rRNA copies
HFLCs, 16 weeks	57.6 ± 10.2	Undetectable (n=4)
cBAL08	61	$2.7 *10^4$ (n=1)
cBAL09	30	Not determined
cBAL20	38	Undetectable (n=1)
cBAL21	31	Undetectable (n=1)
cBAL29	42	$2.4 *10^5 \pm 1.9 *10^5$ (n=3)
cBAL111	Immortal	$2.6 *10^9 \pm 3.2 *10^8$ (n=4)

Characterisation of cBAL111 in vitro

The organisation of the cBAL111 cell layer changed during culturing; the cells displayed a cubic shape at day 15 (Fig. 1C and D) instead of the more spindle shape at day 2 (Fig. 1 A and B). The cBAL111 cultures were positive for the hepatocyte markers GS, CK 18 and albumin as well as the cholangiocyte marker CK 19 at day 2 and day 15 after seeding (GS and albumin are shown at day 2 and CK18 and CK 19 are shown at day 15 in Fig. 1). The CK18 staining was predominantly around the nucleus reaching into the cytoplasm, which is similar to the CK18 staining of primary human hepatocytes that are dedifferentiating *in vitro* [1]. These data suggest that under these conditions the cBAL111 cells resembles cells with progenitor characteristics rather than fully differentiated hepatocytes.

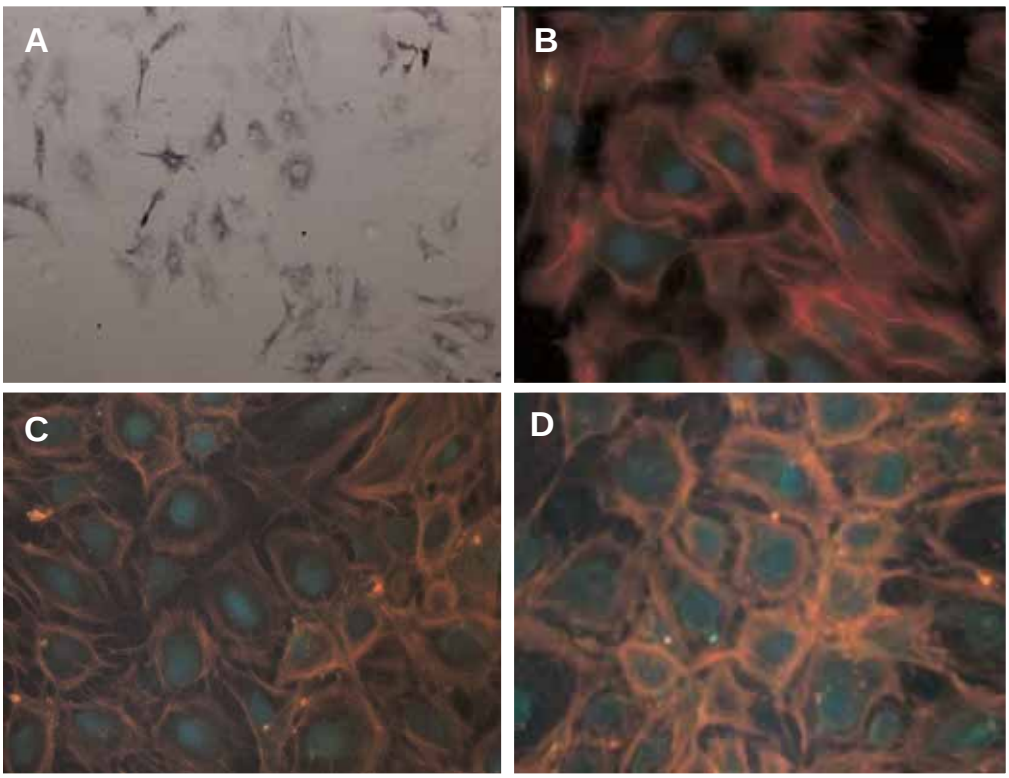

***Figure 1**: Immunostainings of cBAL111 cells in vitro. The cells were stained using antibodies against GS (A) (blue, 40x magnification, 2 days in culture), albumin (B) (green, 400x magnification, 2 days in culture) and CK18 (C) or CK19 (D) (both green, 400x magnification, 15 days in culture). In the CK18, CK19 and albumin staining, cells were further visualized by phalloidin, binding to actin (red), and DAPI, binding to the nuclei (blue).*

Because we recognized a transition in the organisation of the cell layer of cBAL111 cells, we tested their *in vitro* functionality at 2 days of culture, when the cells displayed a spindle like morphology and the culture was not confluent and at day 15, when cells were more cubic and confluence was reached. There was a trend of increased expression of markers for mature hepatocytes (albumin and urea production, transferrin, AAT) with culture time and decreased expression of markers for immature hepatocytes (GSTπ and AFP) (Table 3). The *in vitro* functionality of cBAL08 after reaching confluence was comparable with that of cBAL111, particularly at day 15; no significant difference was detected between cBAL08 and cBAL111 at day 15 for urea and albumin production and the mRNA levels of Albumin, AAT, Transferrin, GSTπ and AFP differed less than 2.5-fold (Table 3). So, the immortalisation of cBAL08 maintained the investigated hepatic functions.

To investigate the putative tumorigenicity of cBAL111, the cells were seeded in soft agar. The cBAL111 cells were not able to form colonies in soft agar, where HepG2 as positive control, formed 61 ± 21 colonies from 5000 cells. The inability of cBAL111 cells to

grow in an anchorage independent way, is an indication that cBAL111 cells are not tumorigenic.

Comparison of cBAL111 with other hepatic cell lines and mature hepatocytes

The novel *in vitro* immortalised fetal hepatocyte cell line cBAL111 was subsequently compared with the hepatoblastoma derived cell line HepG2, the conditionally immortalised hepatocyte cell line NKNT-3 and primary (mature) human hepatocytes two days after seeding. HepG2, cBAL111 at day 15 and NKNT-3 cells, both un-reverted and reverted for immortalisation, synthesized urea, at a level 11-32 fold lower than mature hepatocytes (Table 3). The HepG2 cells and to a lesser extent the cBAL111 cells produced albumin in contrast to NKNT-3 cells.

In cBAL111 and NKNT-3 cells, the mRNA levels of albumin, AAT and transferrin, (markers of hepatocyte differentiation) were less than 1% of the corresponding levels in human liver. HepG2 cells showed mRNA levels for albumin, AAT and transferrin that were comparable to human liver. As markers associated with immature hepatocytes, AFP mRNA levels of cBAL111 and NKNT-3 cells were comparable to the *in vivo* level, while the GST π mRNA levels were 10-44 times higher. In HepG2 cells, mRNA levels for AFP were 1000-fold higher than the *in vivo* levels and GST π levels were undetectable. In summary cBAL111 at day 15 was positive for all tested hepatocyte parameters, whereas HepG2 and NKNT-3 cells both lacked one function.

cBAL111 cells differentiate into functional hepatocytes in murine liver

To determine whether cBAL111 cells have the potential for hepatic differentiation, the cells were marked with GFP by lentiviral transduction and transplanted in the spleen of 4 immunodeficient mice. Nine and 34 days after transplantation, GFP expressing cells were detected in the murine spleen (results not shown) and liver (Fig. 2). The majority of these cells exhibited an elongated morphology, however a small number of cells (~1%) had the morphological characteristics of hepatocytes, given their cuboid appearance. No differences were observed between the livers harvested at nine and 34 days after transplantation. The GFP positive cells were confirmed to be from human origin by immunohistochemistry using a human specific antibody binding to mitochondria (Fig. 2). Immunohistochemistry using an antibody against vimentin, a marker for dedifferentiated and mesenchymal cells [18], indicated a high expression in cBAL111 *in vitro* (data not shown) and in the elongated cBAL111 cells found in the mouse liver, whereas the cBAL111 cells with cuboid appearance did not or hardly expressed vimentin (Fig. 2). Furthermore these GFP positive hepatocyte-looking cells with cuboid morphology were indistinguishable from the surrounding mice hepatocytes with regards to CPS expression, which is expressed periportally [5]. In contrast,

***Table 3**: Hepatic functions of different hepatic cell lines and primary mature human hepatocytes (Mat Hep). MessengerRNA levels are expressed as a percentage of the mean mRNA levels of the two liver samples. ND = not determined*

Function / Cell	Hepatic function		mRNA levels Mature hepatic marker			mRNA levels Immature hepatic marker	
	Albumin production (ng/h/mg protein)	Urea production (nmol/h/mg protein)	Albumin	Transferrin	AAT	GSTπ	AFP
Mat Hep	37.7 ± 7.7	91.5 ± 33.7	130	245	121	535	ND
cBAL08	0.3 ± 0.4	4.0 ± 1.6	0.02 ± 0.01	0.20 ± 0.14	0.32 ± 0.14	1000 ± 565	78 ± 61
cBAL111 day 2	Undetectable	Undetectable	0.02 ± 0.01	0.11 ± 0.05	0.02 ± 0.01	1944 ± 1010	122 ± 11
cBAL111 day 15	0.7 ± 0.8	8.0 ± 6.6	0.02 ± 0.01	0.20 ± 0.06	0.05 ± 0.01	1374 ± 671	114 ± 10
HepG2	2.8 ± 0.3	4.7 ± 0.2	63 ± 6	896 ± 110	199 ± 56	Undetectable	93353 ± 13228
NKNT-3 reverted	Undetectable	4.9 ± 9.4	0.24 ± 0.22	0.16 ± 0.12	0.92 ± 1.52	952 ± 1164	117 ± 166
NKNT-3 unreverted	Undetectable	2.9 ± 5.2	0.42 ± 0.66	0.20 ± 0.13	0.26 ± 0.34	4427 ± 5366	65 ± 79

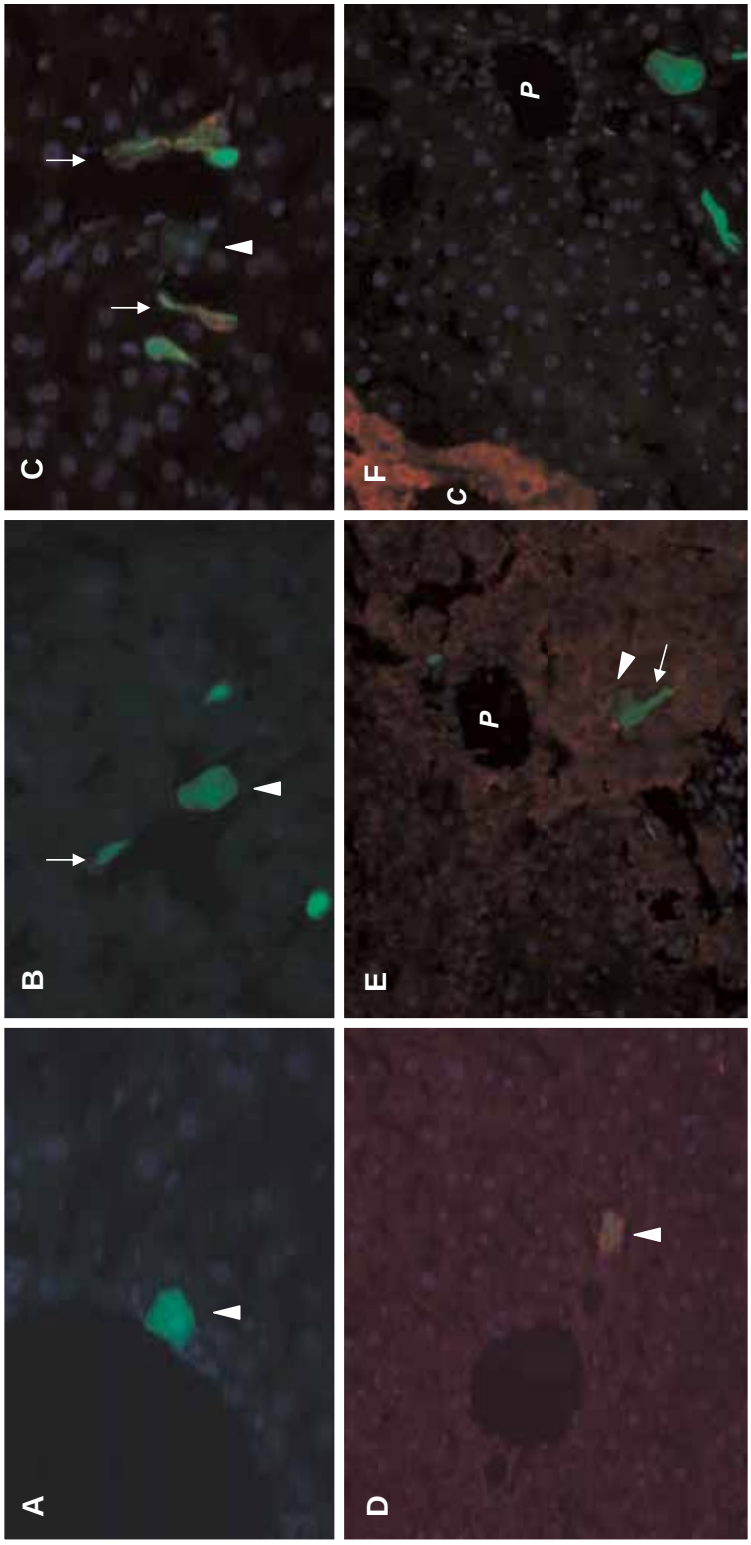

***Figure 2**: Immunofluorescence of mouse livers (40x magnification), harvested 9 days after transplantation of GFP marked cBAL111 cells (green). All nuclei were visualized by DAPI staining (blue). The majority of cells exhibited an elongated morphology (arrows); a small number of cells adapted hepatocyte morphology (arrowheads, A and B). With hepatic differentiation, the cells lost the expression of vimentin, a marker of undifferentiated mesenchymal cells (red) (C). Human origin of the cells was confirmed using an antibody against human mitochondria (red) (D). Hepatocytes originating from cBAL111 are undistinguishable from the surrounding murine hepatocytes in CPS expression (red) (E). No cells originating from cBAL111 were found in pericentral regions; cBAL111 in vivo did not express GS (F). In figure E and F the portal vein is indicated with P, the central vein with C.*

elongated cBAL111 cells did not express CPS. GS expression, which is present *in vitro* (Fig. 1), was absent in the transplanted cells in the periportal areas. No GFP positive hepatocytes were detected in pericentral areas, the site of GS expression in normal liver. A possible explanation for this is that the cells may have entered the liver via the portal vein and engrafted before reaching the pericentral area.

We then tested whether the GFP positive hepatocytes were the result of fusion between human cBAL111 cells and murine hepatocytes [26], by using FISH analysis. The results showed that nuclei reacting to the human probe (Fig. 3 A and D) were negative for the murine probe (Fig. 3 C and F). From the shape of the nuclei and the localization of cells, we concluded that these were cBAL111 derived hepatocytes.

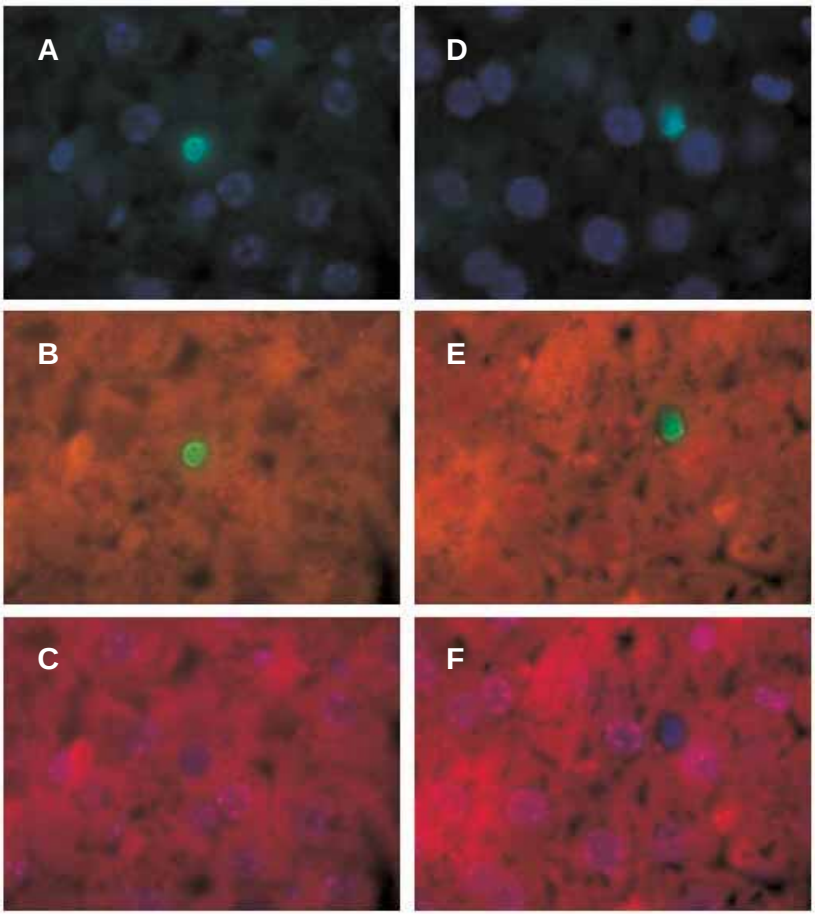

***Figure 3**: FISH analysis (40x magnification) of mouse livers after transplantation of GFP marked cBAL111 cells. The probe hybridizing with human DNA was visualized using a FITC labeled antibody (green, A, B, D and E), the probe hybridizing with murine DNA was visualized using a Texas Red labeled antibody (red, B, C, E and F). All nuclei were counterstained using DAPI (blue A, C, D and F). Picture sets A-C and D-F both show a human cell that is negative for the murine DNA.*

Discussion

In this report we present a novel, clonal, immortalised human fetal liver cell line, cBAL111, that displays hepatocyte-specific functions. *In vitro*, the cBAL111 cell line produced albumin and urea and expressed hepatocyte-specific genes. In addition cBAL111 expressed both cytokeratin 18 and 19 and showed high glutathione S transferase π mRNA levels, but did not show any evidence of growth in soft agar. However, the levels of the hepatic functions showed considerable variation compared with primary mature hepatocytes. *In vivo*, when cBAL111 was transplanted in immunodeficient mice, the cell line showed the potential to fully differentiate into hepatocytes and adapt the zonal expression pattern of CPS.

Human fetal liver cells have been immortalised by telomerase activation before [27], but this is the first report in which this immortalisation strategy was combined with a cloning procedure. In contrast to Wege *et al*. we could not immortalise unselected HFLC preparations [27], although we were able to extend the life span of the cells by about 30%. In our study we selected cells with cuboid morphology from the initial cell preparation and of these selected cells only the cell line with the highest proliferation capacity, cBAL08, became immortal by overexpression of hTERT. The reduced ability of immortalisation in the present study may be the result of differences in transduction levels observed in the two studies. Wege *et al.* used a constitutively active Moloney murine leukemia promoter to drive hTERT expression [27]; we used a tetracycline inducible expression system, stimulated with 1 μM doxycyclin, to drive hTERT expression. Because Moloney murine leukaemia vectors are prone to transduce rapidly dividing cells, this might have resulted in the selection of cells prone to immortalisation.

Our results are in line with the conclusion of Wege *et al.* that telomerase-induced immortalisation of HFLCs does not affect their differentiation potential [27]. After immortalisation, cBAL111 was capable of reaching urea and albumin production rates and the hepatocyte specific mRNA levels comparable to the levels of cBAL08. However, cBAL111 needs prolonged cultivation time to produce albumin and urea.

Furthermore, we found no indications that cBAL111 was malignantly transformed; cBAL111 was not able to form colonies in soft agar and no tumours were found in immunodeficient mice 34 days after transplantation. We conclude that telomerase reconstitution can immortalise fetal human hepatocytes without loss of function and without indications of malignant transformation.

To our knowledge this is the first time that a hTERT-immortalised human fetal liver cell line was evaluated for hepatic function *in vitro*. The clonal origin of the cell line is most important for preservation of the phenotype during long-term culturing. The stability in

phenotype was previously shown for cBAL08 [4]. This is in contrast to cultures of hepatocyte isolates from mature or fetal origin, either immortalised or not.

In addition to the description of cBAL111, we compared its functionality with that of mature human hepatocytes kept under the same culture conditions. It is rather surprising that such comparison is rarely seen in similar studies describing hepatic cell lines. Mature hepatocytes are currently the only cells that meet the criteria for *in vitro* applications and therefore are the gold standard when evaluating alternative hepatic cell lines. The performance of our novel hepatocyte line cBAL111 was also compared with two well-known and widely used hepatic cell lines, NKNT-3 [9] and HepG2 [17]. Admittedly all three cell lines, performed considerably less well compared with primary mature human hepatocytes in all the liver parameters tested in this study. NKNT-3 cell did not produce detectable levels of albumin; HepG2 cells lacked detectable GST π expression. For the cBAL111 cells, all tested parameters were present at day 15 of culture, although still insufficient for most *in vitro* applications. In concordance with the low level of hepatic differentiation, relatively high mRNA levels of AFP and particularly GSTπ were observed in cBAL111. Moreover the cBAL111 cells expressed CK19, a marker for cholangiocytes, and in addition CK18, a marker for hepatocytes, in a pattern characteristic of de-differentiated human hepatocytes. Therefore it can be concluded that cBAL111 cells are not fully differentiated *in vitro* into mature hepatocytes, but rather should be regarded as a progenitor liver cell line that has the full potential to differentiate into hepatocytes.

The liver provides an optimal environment for hepatic differentiation of cBAL111. When cBAL111 cells were labelled with GFP and transplanted into the spleen of immunodeficient mice the cells migrated to the liver. This was already shown with human fetal hepatocytes, both immortalised and freshly isolated [12;27]. However, in this study we showed that following engraftment a number of these cells went to become hepatocytes morphologically indistinguishable from murine hepatocytes. These cells had very low or no expression of vimentin, a marker for mesenchymal cells and for the undifferentiated cBAL111 cells. In addition, the cuboid cells adapted the zonal expression pattern of CPS and GS characteristic for the surrounding cells [5]. With FISH analysis, we excluded the possibility that these GFP positive hepatocytes were the result of fusion between GFP labelled cBAL111 cells and murine hepatocytes. Fusion between host liver cells and transplanted stem cells, specifically haematopoietic stem cells, has been widely reported to account for high rates of transdifferentiation [25;26]. Our experiments confirm that the cBAL111 line is able to differentiate into hepatocytes when the right differentiation stimuli are present.

However, the fact that a significant number of the transplanted cells did not adapt the hepatocyte morphology and expressed high levels of vimentin suggests that either not all cBAL111 cells were equally sensitive to differentiation stimuli, despite the clonal origin of the

cells, or that not all cells were exposed to the same levels of differentiation stimuli due to micro-environmental variations. This requires further investigation.

In conclusion, the development of a cell line that combines both *in vitro* hepatic function and proliferation capacity is important for large-scale applications that depend on *in vitro* hepatic functionality. In this study we present evidence of a novel cell line cBAL111, which is a telomerase immortalised fetal human hepatocyte cell line capable to differentiate into mature hepatocytes *in vivo*. The potential of this novel cell line merits further investigation. The challenge is to define the best possible experimental conditions *in vitro* to mimic as closely as possible the differentiation stimuli present *in vivo* aiming to achieve a high degree of differentiation into mature hepatocytes *in vitro*.

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Chapter 5

The effect of culture time on the *in vitro* hepatic functionality of the immortalised human fetal liver cell line cBAL111

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Abstract

Mature human hepatocytes (MHH) are preferred cells for *in vitro* applications that depend on hepatic function. However, their limited availability and proliferation capacity *in vitro* drives the search for alternative cell sources. Many hepatic cell lines have been developed and various cell types and cell lines are able to differentiate into fully functional hepatocytes *in vivo*. Previously, we observed hepatic differentiation of cBAL111, an hTERT-immortalised human fetal liver cell line after engraftment in the murine liver, but when cultured in monolayer, hepatic functions were maximally 8% of MHHs. The purpose of this study was to determine the effect of culture time on hepatic functions of cBAL111 in relation with its transcriptome.

We investigated a variety of hepatic functions of cBAL111 on day 2 to 5 after seeding, when the cells have adapted to the new culture environment up to day 44 to 47 when net proliferation had stopped. The largest changes in functionality were found between day 2 to 5 and day 15 to 18. Culture time was positively correlated with some aspects of hepatic functionality of cBAL111, most specifically for pregnane X receptor (PXR) expression and galactose elimination. Microarray analysis revealed that four pathways, related to cell proliferation processes, were significantly downregulated between day 2 to 5 and day 15 to 18. Genes related to extracellular matrix, cell-cell interactions and regulation of differentiation processes were upregulated at day 15 to 18.

In conclusion, the results from our analysis showed a trend of increased functionality of cBAL111 with culture time and downregulation of proliferation processes. These findings may contribute to the improvement of *in vitro* conditions to stimulate cell lines to hepatic differentiation.

Introduction

Mature human hepatocytes (MHH) are preferred cells for *in vitro* applications that depend on hepatic function, like pharmacological or toxicological assays and bioartificial liver systems. However, their limited availability and proliferation capacity *in vitro* (1; 2) drives the search for alternative cell sources. Many hepatic cell lines have been developed, either by isolation of cell lines from human liver tumours or by *in vitro* immortalisation of isolated human hepatocytes. In general, these cell lines show sufficient proliferation capacity, but insufficient *in vitro* hepatic functionality. Therefore, the conditions that induce hepatic differentiation *in vitro* need to be identified.

Various cell types and cell lines are able to differentiate into fully functional hepatocytes *in vivo*. Isolated human hepatocytes, from either mature (3) or fetal liver (4) repopulated the murine liver after induction of liver damage. In addition, transplantation of the immortalised human hepatocyte cell line NKNT-3 was able to rescue 90% hepatectomized rats (5) and human fetal liver cells (HFLCs) immortalised by overexpression of human telomerase reverse transcriptase (hTERT) differentiated into fully functional hepatocytes when engrafted into the murine liver (6)(Chapter 4). Moreover, cells from extrahepatic origin, like human adipocytes (7), human pancreatic cells (8) and human cord blood cells (9) have been reported to transdifferentiate into hepatocytes when transplanted in the murine liver.

Cells that have the capacity to differentiate into hepatocytes *in vivo*, are often suggested for *in vitro* hepatocyte applications. However, *in vitro* functionality data of e.g. the above mentioned NKNT-3 cells (10) showed that the function of these cells is insufficient for *in vitro* applications under the current culture conditions. In addition, transdifferentiation of cells from extrahepatic origin *in vitro* is inefficient (7; 11; 12). Thus, *in vivo* the liver directs hepatic differentiation of diverse cells from epithelial or stem cell origin, irrespective their capacity to differentiate to hepatocytes *in vitro.* For hepatic differentiation *in vitro*, identification of the culture conditions that direct this process is needed.

The differentiation state of hepatic cells *in vivo* is the result of a complex network of signals provided by extracellular matrix, the blood-borne factors and cell-cell interactions of both parenchymal and non-parenchymal cells. Actually, hepatocytes *in vivo* do not have a uniform phenotype: depending on their location within the liver lobule, cells show a specific array of metabolic functions (13). Therefore it might be impossible to obtain the total liver function in a single *in vitro* system, if a single cell line is cultured homogeneously. Depending on the *in vitro* application of interest, culture conditions should be optimised to support the function(s) needed. Even though it is likely that specific hepatic functions demand specific culture conditions, some general differentiating stimuli have been identified. These include low serum concentration (14), extracellular matrix (15), 3-dimensional configuration and co-

culturing with non-parenchymal liver cells (16). In addition prolonged culturing time has shown to increase hepatic functionality in HFLCs (17) and the hepatic cell line HepaRG (18).

In a previous study, we developed the hTERT-immortalised cell line called cBAL111 from 16 weeks HFLCs. The cBAL111 cells showed no growth in soft agar and no tumour formation 42 days after transplantation into the spleen of immunodeficient mice which suggests that they are not tumorigenic (Chapter 4). This cell line showed hepatic differentiation in the murine liver, but when cultured in monolayer, hepatic functions were maximally 8% of MHHs (Chapter 4). Under those circumstances the cBAL111 cells expressed not only hepatic markers, but also the bile duct marker CK19. In our previous study, urea and albumin production of cBAL111, showed an increase after prolonged culturing. The purpose of this study was to determine the effect of culture time on hepatic functions of cBAL111 in relation with its transcriptome.

Materials and Methods

Cells and culture conditions

The use of human tissues from adult and fetal origin was approved by the Medical Ethical Committee of the Academic Medical Center, Amsterdam, the Netherlands, subject to informed patient consent.

Human fetal livers were obtained from elective abortions. Gestational age was determined by ultrasonic measurement of the diameter of the skull. Two gestational ages were selected: 16 weeks and 24 weeks. We isolated HFLCs from livers at gestational age of approximately 16 weeks, ranging from 14 to 18 weeks, (16wHFLC) on three independent occasions; in each case four fetal livers were pooled. We isolated HFLCs from livers at gestational age of 24 weeks (24wHFLC) at two independent occasions as described (12; 19).

HFLCs were seeded in DMEM culture medium (Dulbecco's modified Eagle's medium, BioWhittaker) containing 10% heat-inactivated fetal bovine serum (HI-FBS, BioWhittaker), 2 mM L-glutamine (BioWhittaker), 1 µM dexamethason (Sigma), 10 µg/mL insulin, 5.5 µg/mL transferrin, 6.7 ng/mL selenium-X (ITS mix, Life Technology), 100 U/mL penicillin, 100 µg/mL streptomycin (penicillin/streptomycin mix, BioWhittaker) at a density of $3*10^5$ cells/cm^2 in Primaria 6-well plates (BD Falcon).

Primary MHHs were isolated from seven patients undergoing partial hepatectomy, because of metastatic carcinoma. The tumour-free liver tissue used for the hepatocyte isolation ranged in weight between 2 to 10 grams. The hepatocyte isolation method was adapted from the protocol described by Seglen (20) as previously described (12). The MHH cells were cultured in WE culture medium (Williams' E) containing 4% HI-FBS, 2 mM L-

glutamine, 1 µM dexamethason, 20 mU/mL insulin (Novo Nordisk), 2 mM ornithine (Sigma-Aldrich), 100 U/mL penicillin, 100 µg/mL streptomycin.

cBAL111 cells were expanded using DMEM culture medium. The seeding density of cBAL111 cells was 15.000 cells/cm^2, resulting in 50% confluence, unless stated otherwise. During expansion, cells were passaged with a split ratio of 1:2. Medium was refreshed every 2-3 days.

All cells were maintained at 37ºC in a humidified atmosphere (95% air, 5% CO_2).

Set-up hepatic function test

Hepatic function tests of 16wHFLCs (3 isolates), 24wHFLCs (2 isolates) and MHHs (7 isolates) were performed starting from day 2 after seeding. The cBAL111 cells (3 independent cultures) were tested at different time points as indicated. The cultured cells were washed twice using phosphate buffered saline (PBS, NBPI International) and then incubated with test medium consisting of WE culture medium supplemented with 0.5 mM NH_4Cl (Merck), 2.75 mM D-galactose (Sigma), 90 µM lidocaine (Sigma) and 2 mM L-lactate (Sigma). Medium samples were taken immediately from the culture wells, as a reference, and after different times of culturing; 24 hours for the MHHs and 72 hours for the HFLCs and cBAL111. At the end of the test period the cells were washed three times using PBS and stored at –20˚ C for protein determination. Hepatic function tests were performed in triplicate per cell isolate.

Biochemical assays

Urea concentrations were determined using the blood urea nitrogen test (Sigma Chemical Co). Galactose concentrations were enzymatically determined as described (Bergmeyer 1974). Total protein/well was quantified by spectrometry using Coomassie blue (Bio-Rad). Function test parameter rates were determined by calculating the changes in concentration in test medium per hour per mg protein.

Real time RT-PCR

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) reactions were performed to quantify mRNA levels of the genes *Albumin*, *Gluthatione-S-transferase* π (*GST* π), *Hepatocyte Nuclear Factor 4* (*HNF4*), *Multidrug Resistance Protein* 2 (*Mrp2*), *Pregnane X Receptor* (*PXR*), *Transferrin* and, as a loading control, of *18S* ribosomal RNA. RNA was isolated from HFLCs and MHHs after 2 days of culturing and from cBAL111 cultures at different culture periods as indicated. As a reference, RNA was isolated from two 0.2-0.5 gram snap-frozen adult human liver samples. Total RNA isolation and gene-specific RT reactions were performed as described previously (10).

Real-time PCR was performed using 1 µl of cDNA and Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). Primers and reaction conditions are depicted in Table 1. All reactions were carried out in 3mM $MgCl_2$. The thermal cycling profile of the touchdown PCR was as follows: 50°C for 2 min, 95 °C for 2 min, followed by 40 cycles of 94 °C for 1 s, primer annealing at high annealing temperature for 7 s with a 0.5 °C/cycle decrease until low annealing temperature, and

extension for 40 s at 72°C. PCR specificity was verified by melting curve analysis and agarose gel electrophoresis. The mRNA quantification was carried out by using the LinRegPCR programme (21). For each sample the mRNA starting level was normalised for the *18S* rRNA starting level. Finally the normalized mRNA levels of replicate samples, starting from different RNA isolates, were averaged. All mRNA levels are expressed as a percentage of the levels found in mature human liver samples.

Microarrays

RNA isolated from cBAL111 cells at 2 and 15 days of culturing, from 16wHFLCs and MHHs at 2 days of culturing was used for microarray analysis using Agilent 44K Whole Human Genome microarrays. For both time points, microarray analysis was performed in triplicate, using RNA from three independent cultures. RNA of multiple standardized HepG2 cultures was pooled and used as reference material. RNA labelling, hybridisation and array scanning was performed by ServiceXS, Leiden, the Netherlands (www.servicexs.com).

Default settings of the Agilent Feature Extraction pre-processing protocol were used to obtain unnormalized foreground and background intensity values from the raw scans. Exact protocol and parameter settings are described by Agilent Feature Extraction Software User Manual 7.5 (22). Quality control of the Agilent Feature Extraction results was performed using methods available from Bioconductor packages (marray, limma, arrayQuality) (23). Intensities of negative and positive controls were inspected, as well as spatial effects, M/A plots and signal-to-noise distributions. All arrays passed quality control.

The data was first normalized using variance stabilization normalization (24). This was followed by quantile normalization on the common reference channels (Cy5) between the arrays while leaving the log-ratios unchanged (25). To find differentially expressed genes, separate channel analysis was performed between the comparisons of interest using a moderated t-test (26). This test is similar to a standard t-test but uses information of the variation in intensity values in the whole dataset to moderate (shrink) the standard error in order to stabilize inference. The resulting p-values were corrected for multiple testing using a false discovery rate (FDR) adjustment procedure (27).

***Table 1**: Primers and conditions used in RT-PCR analysis*

Gene	primers 5' → 3'		Amplicon size (bp)	Real-time RT-PCR conditions		
				[$MgCl_2$] (mM)	Template dilution	Annealing temp. (°C)
18S rRNA	RT S AS	CGAACCTCCGACTTTCGTTT TTCGGAACTGAGGCCATGAT CGAACCTCCGACTTTCGTTCT	151	3	1000 X	68 ↓ 63
Albumin	RT S AS	ACTTCCAGAGCTGAAAAGCATGGTC TGAGCAGCTTGGAGAGTACA GTTCAGGACCACGGATAGAT	189	4		68 ↓ 63
GST π	RT S AS	AGCAGGTCCAGCAG GTTG GCCAGAGCTGGAAGGAGG TTCTGGGACAGCAGGGTC	333	5	10 X	70 ↓ 63
HNF4α	RT S AS	CACTCCAACCCCGCCCCTC CCGGGTGTCCATACGCATCCT CAGGTTGTCAATCTTGGCC	321	3		68 ↓ 63
Mrp2	RT S AS	GACGATGATGGTGAAGACAGGAG AGCACCGACTATCCAGCATCTC ATCCGGCCTGTGGGTGTTGTG	278	2		68 ↓ 63
PXR	RT S AS	CATGTGGGGCAGCAGGGAGAAG CGCCTGCGCAAGTGCCTGGAG GTCGGCTGGGGGTTTGTAGTTC	420	2		68 ↓ 63
Transferrin	RT S AS	CCAGACCACACTTGCCCGCTATG GAAGGACCTGCTGTTTAAGG CTCCATCCAAGCTCATGGC	310	2		68 ↓ 63

The last part of the analysis focused on finding differentially expressed pathways. We obtained pathways from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (28) using annotation from the R-packages 'hgug4112a' and 'KEGG'. A Wilcoxon test was used to test if a pathway is enriched with highly ranked genes. Genes were ranked by their t-value obtained with the moderated t-test described previously. When two or more probes on the array point to the same gene, the probe with the highest average intensity in the Cy3 channel was taken. The resulting p-values are adjusted for the FDR. Our R-scripts are available upon request.

Results

Proliferation of cBAL111

To investigate the relation between cell density and hepatic functionality, we monitored the growth of cBAL111 without passaging. After seeding at low density, the protein content per well increased and after approximately 33 days of culturing a plateau was reached (Fig. 1). Based on these data, we decided to analyse the cells for functionality at 4 time points: from

day 2 to 5, when the cells have adapted to the new culture environment after passaging and start proliferating; from day 15 to 18, when cells are proliferating steadily; from day 30 to 33, when cells are reaching the plateau phase and from day 44 to 47, when proliferation is inhibited because of contact inhibition.

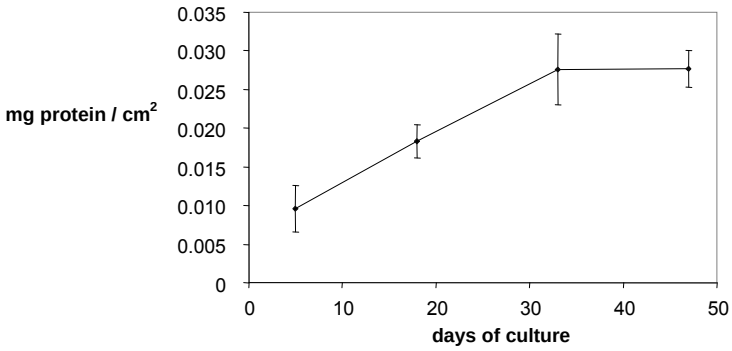

***Figure 1**: Growth of cell line cBAL111, determined as mg protein / cm^2 (n=3). Values are given as mean ± SD.*

Hepatocyte specific function and cell density

We investigated the relation between culture time and hepatic functionality of cBAL111. Urea production and galactose elimination were determined at four different time points after seeding (Fig. 2 and 3 respectively) and both showed a trend of increased functionality between the first and second test period. No differences were found between the last three time points for both parameters. The maximum urea production of cBAL111 was 3% of the MHHs, while 16wHFLCs and 24wHFLCs produced 21% and 50% urea, respectively, compared to MHHs (Fig. 2). The maximum galactose elimination of cBAL111 was 10% of the MHHs; 16wHFLCs and 24wHFLCs showed 17% and 40% of the galactose elimination of MHHs, respectively (Fig. 3).

To evaluate the effect of culture time on the expression of hepatic genes Mrp2 and GST π as well as nuclear hormone receptors HNF4 and PXR in cBAL111, the mRNA levels were determined using real-time RT-PCR at day 5, 18, 33 and 47 after cell seeding. For comparison the mRNA levels were also determined in adult human liver, MHHs, 16wHFLCs and 24wHFLCs. Of the genes that are positively related to hepatic differentiation, *i.e*. *HNF4*, *PXR* and *Mrp2*, only HNF4 and Mrp2 mRNA were detectable in cBAL111 at the earliest time point. PXR mRNA was detected at day 18, 33 and 47 at 10% of mature liver (Fig. 4A). HNF4 mRNA levels showed a sharp increase at day 18, up to 20% of mature liver and was around 2% at the other time points. The mRNA levels of GST π, marker for immature hepatocytes,

were at least 11 times higher than mature liver at all time points (Fig. 4B) and remained stable.

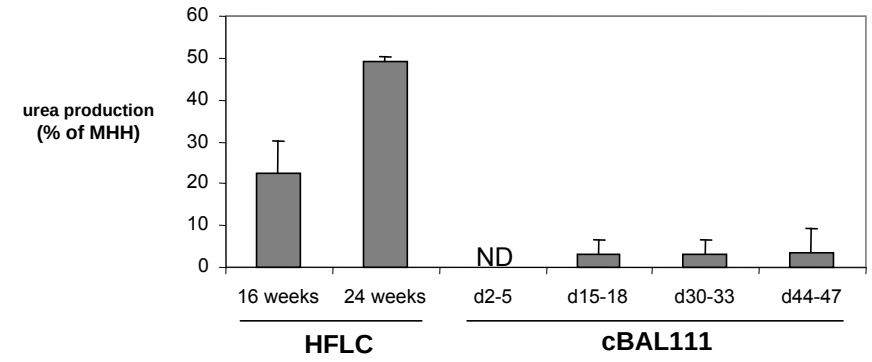

***Figure 2**: Urea production rate of cBAL111 cells during prolonged culturing (n=3), compared with HFLCs from gestational age of 16 weeks (n=3) and 24 weeks (n=2) and MHHs (n=8). The urea production rates are given as a percentage of MHHs cultured under the same conditions (91.5 ± 33.7 nmol/h/mg protein). Values are given as mean ± SD. ND: not detectable.*

Microarray analysis of cBAL111

To gain insight in the genetic programmes that are involved in the changes in cBAL111 at increasing culture time, we performed microarray analysis on RNA samples of cBAL111 at day 2 and day 15 after seeding. 16wHFLCs and MHHs cultured for 2 days were also included in this analysis.

Cluster analysis of all data points resulted in the dendrogram (Fig. 5) showing clustering according to the biological origin of the samples. The triplicates of the different

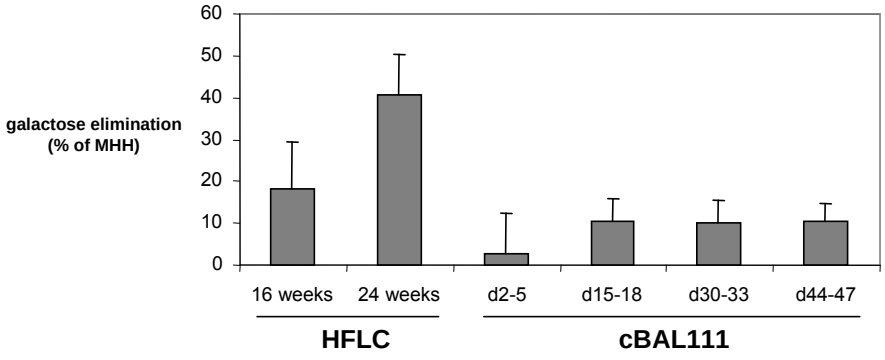

***Figure 3**: Galactose elimination rate of cBAL111 cells during prolonged culturing (n=3), compared with HFLCs from gestational age of 16 weeks (n=3) and 24 weeks (n=2) and MHHs (n=5). The galactose elimination rates are given as a percentage of MHHs cultured under the same conditions (577 ± 442 nmol/h/mg protein). Values are given as mean ± SD.*

samples show high levels of similarity and, except the cBAL111 day 2 group, the triplicates cluster together before they cluster with other groups. This dendrogram shows also high similarity between the two cBAL111 groups and between the primary 16wHFLC and MHH groups.

To verify the results of the microarray analysis, we compared the calculated relative mRNA levels obtained for the genes *Albumin*, *GST π* and *Transferrin* with real-time RT-PCR outcomes (Fig. 6). This comparison showed similar trends for both analyses, although the range of mRNA levels determined by RT-PCR was more pronounced compared to the microarray data.

The microarray analysis revealed 415 genes that were more than 2-fold downregulated at day 15 when compared to day 2 of culture; 101 genes were more than 2-fold upregulated at day 15 when compared to day 2 of culture. Table 2 shows the top 10 genes at both ends of this spectrum. The majority (7/10) of the top-ten genes with relative high transcript levels at day 2 are involved in mitosis or cell cycle regulation, *i.e.* the genes encoding for CENPF, HCAP-G, TOP2A, SMC4L1, KNTC2, CDC45L and KIF20A. Of the top ten genes that showed a high relative transcript level at day 15, five have a regulatory function, *i.e.* genes encoding CUTL2, EDN2, NAV1, KIAA1199 and GEM and are associated with cell-cell interaction or differentiation. Furthermore one hepatocyte-specific enzyme, GSTA1, was upregulated. The top 30 genes that showed a high relative transcript level at day 15 (only top ten is shown), included 9 other genes involved in extracellular matrix production and/or cell-cell interactions, *i.e.* the genes encoding elastin, fibronectin, collagen and biglycan.

In addition to individual genes, the microarray analysis also revealed 4 pathways that were significantly downregulated between day 2 and day 15 of culture (an fdr value below 0.2). These included Biosynthesis of steroids (KEGG pathway reference “00100”, fdr 0.00413), Cell cycle (KEGG pathway reference “04110”, fdr 0.08300), Oxidative phosphorylation (KEGG pathway reference “00190”, fdr 0.08300) and Ribosome (KEGG pathway reference “03010”, fdr 0.12955).

Because our research was specifically aimed at hepatic differentiation, we selected 26 key genes, known to be related to hepatic differentiation, and compared the transcript levels of these genes between cBAL111 at day 2 and day 15, MHHs and HFLCs (Table 3). Most of these genes are related to mature liver, except for AFP, ONECUT1 and SOX17, which are more related to the embryonic liver. The differential expression was never more than two-fold between the cBAL111 samples for this subset of genes. Four genes were differentially expressed between cBAL111 cells at 2 and 15 days, *i.e.* two enzymes involved in gluconeogenesis (G6PC2 and PCK1) and two transcription factors (ONECUT1 and SOX17). These genes were transcribed at relatively higher levels in cBAL111 cells at 2 days.

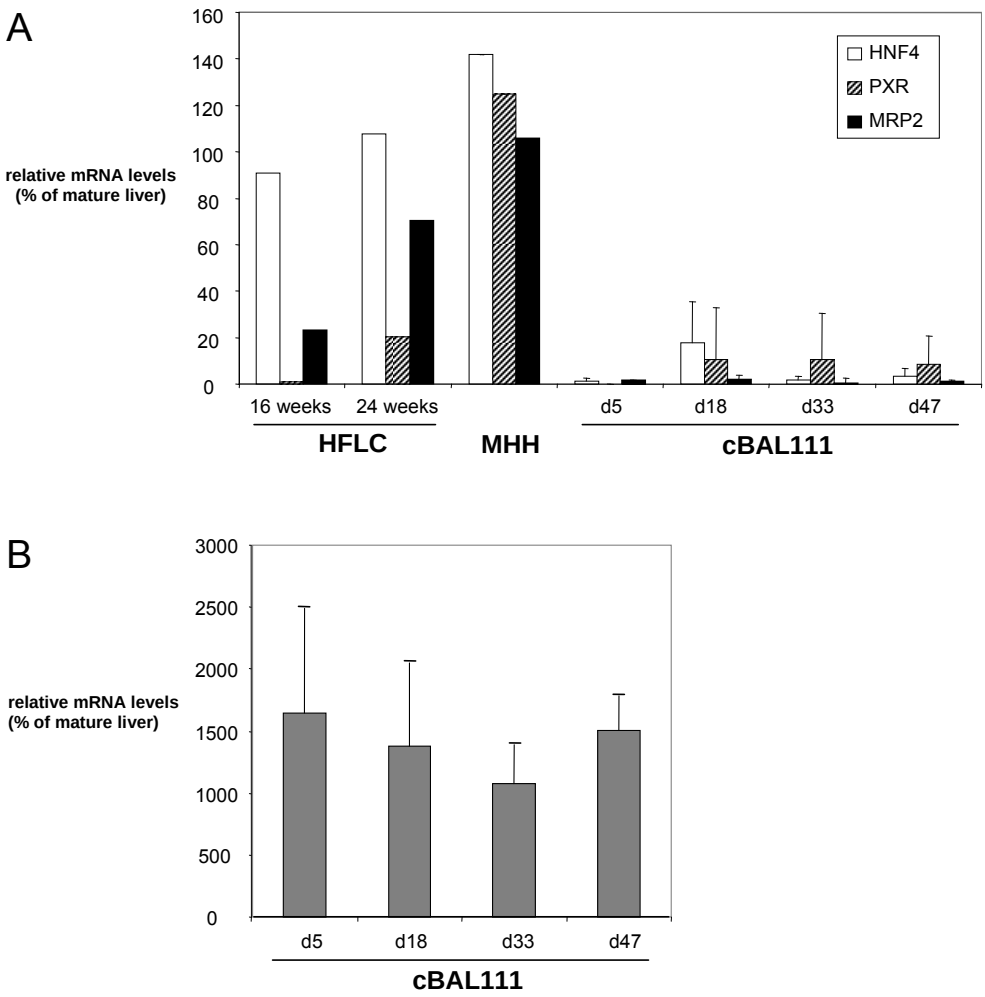

***Figure 4**: The average relative mRNA levels of genes associated with hepatic differentiation, HNF4, PXR and Mrp2 (A), and a gene associated with immature hepatocytes, GST π (B), were measured in cBAL111 during prolonged culturing (n=3) and compared with the mRNA levels of HFLCs from gestational age of 16 weeks (n=2) and 24 weeks (n=2). All mRNA levels are given as a percentage of those of MHHs (n=2) cultured under the same conditions. Values are given as mean ± SD, for n=2 results no SDs are given.*

ONECUT1 is related to differentiation of bile duct cells and is expressed in undifferentiated hepatocytes (29), whereas SOX17 is thought to be a regulator of endoderm formation and a transcription activator of FOXA2, which directs liver development at an early stage (30). There was a trend of higher expression of plasma proteins in cBAL111 at day 15 compared to cBAL111 at day 2.

Clearly, most genes were not equally expressed between cBAL111 (day 2 and day 15) and MHHs, except for GS, G6PC2, AFP, CYP7A1, SOX17, CYP2B6 and, only for cBAL111 at day 15, CYP1A2. These genes were also differentially expressed between cBAL111 (day 2 and day 15) and HFLCs, except for AFP and SOX17 for cBAL111 at day 2. The number of differentially expressed genes was larger between cBAL111 (day 2 and day 15) and MHH as between cBAL111 (day 2 and day 15) and HFLCs (20 and 19 genes *versus* 9 and 11 genes) in this subset of 26 genes. This trend is not valid for the whole genome, as can be deduced from the cluster dendrogram (Fig. 5).

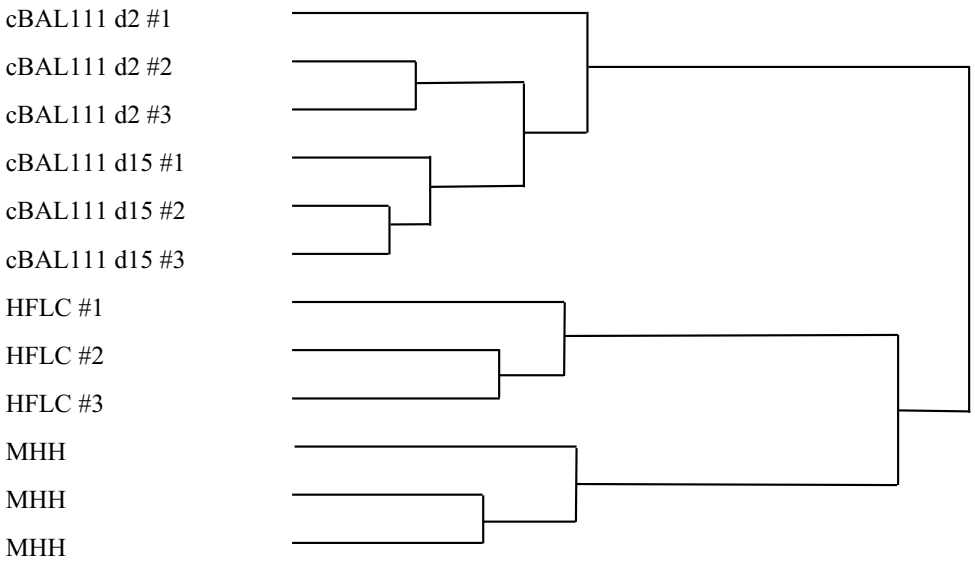

***Figure 5**: Cluster dendrogram based on the genome wide expression profiles obtained with the microarrays. Hierarchical clustering was used with pearson correlation as distance measure and complete linkage as agglomeration method.*

Discussion

In this study we investigated the effect of culture time on the hepatic functions of cBAL111, an immortalised human fetal liver cell line. There was a positive trend of the hepatic functionality of cBAL111 with culture time, as monitored by PXR expression and galactose elimination, and probably also urea production.

We investigated a variety of hepatic functions at four different time points during culture, from day 2 to 5, when the cells have adapted to the new culture environment after passaging and start proliferating; from day 15 to 18, when cells are proliferating steadily; from day 30 to 33, when cells are about to reach the plateau phase and from day 44 to 47 when no net proliferation is observed. The largest changes in functionality were found between the first two test periods and, except for mRNA levels of HNF4, no differences were found between the last three test periods. From day 2 to 5, no urea production was measurable and only 3% galactose elimination compared to MHHs. For the urea production, the trend to increased function between the first and second test period, may include technical aspects, since the low cell/medium ratio can result in urea concentrations below the limit of detection. The increased functionality between day 2-5 and day 15-18 was indeed previously observed for albumin and urea synthesis and mRNA levels of transferrin and AAT (Chapter 4). However, the functionality did not reach levels necessary for *in vitro* applications; biochemical parameters did not exceed 10% of the functionality of MHHs.

A trend to a positive relation between culture time and functionality may be the result of increasing cell density, which has been linked to increased hepatic functionality *in vitro* before (31). Cell-cell interactions provide important stimuli for hepatic differentiation, *e.g*. the addition of epithelial cells to human hepatocyte cultures can rescue the hepatocyte phenotype (reviewed by (32)). Previous immunocytochemical analyses of cBAL111 showed that the cells displayed a spindle-like morphology with very limited cell contacts at day 2, but that a cuboid morphology with close cell contacts was reached at day 15 (Chapter 4). The low functionality at day 2-5 could also be the result of the cells still reorganising after being passaged and / or a relatively low disposition of extracellular matrix. Extracellular matrix components excreted by hepatocytes, can provide differentiation stimuli to hepatocytes in an autoregulatory loop (15).

Hepatic functionality being stable between the second and the fourth test period, even though cell density is still increasing, could be the result of a threshold in the stimuli that cell-cell interactions can provide, which might have been reached at the cell density at day 15. Clearly, the further increase in cell density after day 15, does not contribute to further differentiation.

***Table 2**: Genes differentially expressed between cBAL111 at day 2 and day 15 of culture, 10 genes with highest expression at day 2 and 10 genes with highest expression at day 15 .*

Accession #	Description	P value	Fold change (day 2 vs. day 15)	Function - Processes
NM_016343	Centromere protein F (CENPF)	3,49E-04	8.15	Nuclear protein associating with the centromere-kinetochore complex. Potential role in chromosome segregation during mitotis.
NM_022346	Chromosome condensation protein G (HCAP-G)	1,41E-05	5,73	Nuclear protein with role in chromosome condensation and mitosis.
NM_005891	Acetyl-Coenzyme A acetyltransferase 2 (ACAT2)	9,61E-15	5,12	Cytoplasmic enzyme involved in lipid metabolism.
NM_001067	Topoisomerase (DNA) II alpha (TOP2A)	4,30E-06	4,98	Nuclear enzyme involved in chromosome condensation, chromatid separation, and the relief of torsional stress occurring during transcription and replication.
NM_005496	SMC4 structural maintenance of chromosomes 4-like 1 (yeast) (SMC4L1)	1,15E-06	4,42	Nuclear protein, involved in mitotic chromosome condensation and sister chromatid cohesion.
THC2199911	Unknown	1,71E-05	4,34	Unknown.
NM_006101	Kinetochore associated 2 (KNTC2)	1,93E-07	4,21	Nuclear protein involved in spindle checkpoint signaling during cell division.
BC041395	Similar to diaphanous homolog 3 (Drosophila)	5,72E-06	4,06	Unknown.
NM_003504	CDC45 cell division cycle 45-like (S. cerevisiae) (CDC45L)	4,22E-11	4,04	Nuclear protein essential for the initiation of DNA replication.
NM_005733	Kinesin family member 20A (KIF20A)	2,10E-05	4,00	Protein associated with Golgi apparatus and microtubule and involved in membrane traffic and in cell division during cleavage furrow formation and cytokinesis.
NM_015267	Cut-like 2 (Drosophila) (CUTL2)	5,47E-06	0,33	Transcription factor potentially involved in proliferation and differentiation.
A_32_P214860	Unknown	8,02E-09	0,32	Unknown.
NM_001956	Endothelin 2 (EDN2)	5,09E-05	0,32	Excreted peptide involved in cell-cell signalling and with vasoactive functions. Expression results in mitogenic effect in ovarian cancer cell lines.

Accession #	Description	P value	Fold change (day 2 vs. day 15)	Function - Processes
A_23_P395321	Unknown	2,03E-08	0,29	Unknown.
NM_145740	Glutathione S-transferase A1 (GSTA1)	4,40E-06	0,29	Cytoplasmic enzyme involved in detoxification by conjugation of glutathione
NM_020443	Neuron navigator 1 (NAV1)	7,03E-04	0,28	Cytoskeletal protein, expressed in many tissues and potentially involved in differentiation. The exact function is unknown.
NM_014747	Regulating synaptic membrane exocytosis 3 (RIMS3)	5,82E-04	0,28	Protein essential for normal neurotransmitter release, but also expressed in unknown localisation liver.
NM_007281	Scrapie responsive protein 1 (SCRG1)	2,62E-08	0,27	Extracellular protein associated with neurodegenerative changes observed in transmissible spongiform encephalopathy.
NM_018689	KIAA1199	5,64E-06	0,18	Cytoplasmic protein with potential role in cellular mortality of normal human cells; counters cell immortalisation and carcinogenesis.
NM_005261	GTP binding protein overexpressed in skeletal muscle (GEM)	5,16E-13	0,15	Plasma membrane protein with potential role as a regulatory protein in receptor-mediated signal transduction, also expressed in liver.

Table 2 continued

***Table 3**: Transcript levels of hepatic key genes of cBAL111 at day 2 and day 15 compared to MHHs and HFLCs.*

Accession #	Description	Fold change in transcript levels				
		d2 vs d15	d2 vs MHH	d15 vs MHH	d2 vs HFLC	d15 vs HFLC
Amino acid metabolism / urea cycle						
NM_001875	Carbamoyl-phosphate synthetase 1, mitochondrial (CPS1)	1.464	0.083*	0.057*	0.342	0.234*
NM_000531	Ornithine carbamoyltransferase (OTC)	0.907	0.176*	0.194*	0.542	0.598
NM_054012	Argininosuccinate synthetase (ASS), transcript variant 2	0.836	0.172*	0.206*	0.526	0.629
NM_000048	Argininosuccinate lyase (ASL)	1.037	0.181*	0.174*	0.554	0.534*
NM_000045	Arginase, liver (ARG1)	0.860	0.015*	0.017*	0.416	0.484
NM_002065	Glutamate-ammonia ligase (glutamine synthase) (GS)	0.926	0.831	0.897	0.765	0.826
I_959813	Tyrosine aminotransferase (TAT), nuclear gene encoding mitochondrial protein	1.115	0.354*	0.318*	1.210	1.085
Carbohydrate metabolism						
NM_021176	Glucose-6-phosphatase, catalytic, 2 (G6PC2)	1.273*	0.786	0.618	0.775	0.608
NM_002591	Phosphoenolpyruvate carboxykinase 1 (soluble) (PCK1)	1.604*	0.242*	0.151*	0.786	0.490
Cytoskeleton						
THC1581566	HUMKER18A cytokeratin 18 (CK18)	0.862	0.323*	0.375*	0.196	0.227
Plasma protein						
NM_000477	Albumin (ALB)	0.806	0.016*	0.020*	0.020*	0.025*
NM_000371	Transthyretin (prealbumin, amyloidosis type I) (TTR)	0.791	0.010*	0.012*	0.103*	0.130*
NM_001063	Transferrin (TF)	0.665	0.054*	0.082*	0.202*	0.303*
NM_001134	Alpha-fetoprotein (AFP)	0.977	1.275	1.306	0.108*	0.111*
Protein processing						
AF113676	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antitrypsin) (SERPINA1)	0.861	0.112*	0.130*	0.170*	0.198*
Steroid metabolism						
NM_000780	Cytochrome P450, family 7, subfamily A, polypeptide 1 (CYP7A1)	0.876	0.731	0.834	1.279	1.459
NM_000039	Apolipoprotein A-I (APOA1)	0.913	0.071*	0.078*	0.202*	0.222*
Transcription regulation, liver development						
NM_000545	Transcription factor 1, hepatic; LF-B1, hepatic nuclear factor (HNF1A)	1.088	0.259*	0.238*	0.482*	0.443*
NM_021784	Forkhead box A2 (FOXA2), transcript variant 1	1.213	0.233*	0.193*	0.472	0.393
NM_004498	One cut domain, family member 1 (ONECUT1)	1.667*	0.263*	0.158*	0.430	0.258*
NM_004364	CCAAT/enhancer binding protein (C/EBP), alpha (CEBPA)	0.939	0.118*	0.126*	0.537	0.573
NM_022454	SRY (sex determining region Y)-box 17 (SOX17)	1.221*	2.131	1.744	2.247*	1.840
Xenobiotic metabolism						

NM_000761	Cytochrome P450, family 1, subfamily A, polypeptide 2 (CYP1A2)	1.177	0.635*	0.539	1.038	0.882
NM_000767	Cytochrome P450, family 2, subfamily B, polypeptide 6 (CYP2B6)	1.011	0.737	0.729	0.987	0.976
NM_000771	Cytochrome P450, family 2, subfamily C, polypeptide 9 (CYP2C9)	1.138	0.078*	0.069*	0.917	0.806
NM_017460	Cytochrome P450, family 3, subfamily A, polypeptide 4 (CYP3A4), transcript variant 1	0.742	0.020*	0.027*	0.140*	0.188*

Table 3 continued

* = P < 0.05.

Besides describing the change in functionality during culture, microarray analyses were performed to identify the genetic programs that underlie the functional changes. The results obtained by microarray analysis were validated by determining mRNA levels of a selected group of genes by RT-PCR. Both analyses showed comparable results, although differences in mRNA levels between cell types as determined by RT-PCR were more pronounced compared to those established using microarrays.

Because the most functional changes were observed between day 2 to 5 and day 15 to 18, we investigated the cBAL111 transcriptome at day 2 and day 15 of culture by microarray analysis. Unfortunately, the induction of hepatic functions was hardly conclusive upon inspection of the genes differentially expressed more than 2-fold between day 2 and day 15. The microarray analysis revealed, however, that four regulatory pathways were downregulated with increasing culture time. These four are related to cell proliferation processes: the cell cycle, the biosynthesis of steroids and proteins, as well as the oxidative phosphorylation to ensure sufficient ATP availability. Furthermore, seven of the top-ten genes with the highest relative expression at day 2 are involved in mitosis, again underlining the focus on cell proliferation in that phase. Our microarray data showed that the downregulation of cell proliferation processes started well before growth inhibition. The upregulation of GSTA1, a member of the alpha class glutathione S-transferases most abundantly present in the liver and involved in detoxification by glutathione conjugation (33), suggested that hepatic differentiation was indeed stimulated. The trend to hepatic differentiation is supported by the increased plasma protein expression, although not significant.

To investigate the mechanisms underlying the transcriptome changes during the culture of cBAL111, we investigated a number of hepatic transcription regulators. Our microarray analysis did not show differential expression of transcription factors thought to be dominant in the final stages of hepatic maturation, like HNF1A and CEBPA (34), although the RT-PCR did show a relative increase in HNF4 transcript levels at day 15. In contrast, we observed a significant downregulation of ONECUT1 and SOX17, which are more related to embryonic liver. The top ten genes with high relative expression at day 15, included five other genes that are associated with governing differentiation, for instance KIAA1199, which is thought to counteract immortalisation processes (35). Thus, particularly transcription regulators from the early phases of liver development may be implicated in the observed changes in the transcriptome in cBAL111 during culturing. This may be associated with the increase in extracellular matrix and close cell contacts, as we found that 37% of the top 30 genes with relative high transcript levels at day 15 are involved in extracellular matrix deposition and/or cell-cell interactions.

Previously, hepatic differentiation has been monitored using microarrays. This was carried out with liver epithelial progenitor cells which were differentiated using sodium butyrate (36) and with HBC-3 liver stem cells which were differentiated using DMSO (37). Here we present for the first time whole genome expression analysis with culture time as a hepatic differentiation stimulus. As expected, these studies, our current results included, showed a decrease in proliferation related processes. The study of Plescia et al. focused on the loss of stem cell properties during the DMSO induced differentiation process. Aninat *et al.* in 2005 presented a cell line, HepaRG, which exhibits hepatic differentiation after two weeks at confluency (38). In this cell line various nuclear hormone receptors, CYPs, phase 2 enzymes and other liver-specific functions were upregulated. In concordance, our results did suggest an increase in PXR expression. This nuclear hormone receptor drives the expression of a diverse array of detoxifying genes (39). However, our microarray analysis did not reveal upregulation of the same genes.

In conclusion, the results from our analysis showed a tendency to hepatic differentiation of cBAL111 with culture time and downregulation of proliferation processes. These findings may contribute to the improvement of *in vitro* conditions to stimulate cell lines to hepatic differentiation. For instance, overexpression of KIAA1199 might further downregulate proliferation and stimulate hepatic differentiation. Providing cBAL111 cells with appropriate extracellular matrix might improve hepatic differentiation. This may also be applicable to other hepatic cell lines, perhaps only in cell lines showing contact-inhibition. Still, our results show that downregulation of proliferation is not sufficient to explain the observed hepatic differentiation *in vivo*. More research is needed to improve the *in vitro* conditions to stimulate cell lines to hepatic differentiation.

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Chapter 6

Increased reproducibility of quantitative reverse transcriptase-PCR

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Notes and Tips

Real-time reverse transcriptase-PCR (RT-PCR) is increasingly used as a rapid and sensitive method of quantifying mRNA levels of selected genes for molecular diagnostics and transcriptional profiling. This technique relies on the monitoring of fluorescent PCR product accumulation after each PCR cycle [1,2]. Fluorescence of PCR products is most cost-effectively established by the use of the dye SYBR® Green I, of which fluorescence intensity increases linearly over three orders of magnitude upon associating to double stranded DNA [3,4]. Alternatively, accumulating PCR products can be detected by using fluorescent probes [5-8]. Real-time PCR is highly reproducible, providing that primers, probes and PCR conditions are well-designed and critically assessed to prevent amplification of nonspecific PCR products and primer-dimer formation [9,10]. However, the RT reaction that precedes the PCR reaction is recognized as the step introducing the largest experimental variation in real-time RT-PCR [11,12]. The lack in reproducibility is most evident for real-time RT-PCR of low expressed genes in combination with the use of SYBR® Green I [12,13]. The reproducibility of the RT reaction depends, at least partly, on the priming strategy, the sample and the enzyme system used [10-12]. Moreover, the optimal RT conditions may vary among different genes. We describe here an improvement of the RT-PCR reaction conditions using SYBR® Green I, that yields a constant higher reproducibility in real-time RT-PCR of genes that are expressed at various levels in different samples. In addition we will describe for the first time the character of the relation between the relative mRNA starting levels and the variability of RT-PCR data.

In order to maintain variation between RNA samples we used different samples that expressed target genes over a broad range. The samples analyzed in our experiment comprised two sets of related origins: two liver samples harvested, in accordance with the ethical standards of the institutional committee on human experimentation, from different patients who had undergone liver resection and three samples of cBAL58, a clonal derivative from the human hepatic cell line NKNT-3 [14] that had undergone different treatments: complete reversion of immortalisation, partial reversion of immortalisation and no reversion of immortalisation. Reversion of immortalisation was reported to stimulate the expression levels of liver specific genes. The two sample sets vary to a great extent in expression of liver genes: *alpha-1 antitrypsin* (*AAT*), *albumin*, *transferrin* are significantly higher expressed in liver samples as compared to cBAL58 cells whereas dedifferentiation marker *glutathione S-transferase* π *subunit* (*GST*π) is thought to be expressed at a higher or similar level in cBAL58 cells. Total RNA was isolated from the two 0.2-0.5 g snap-frozen liver samples and cBAL58 cultures comprising 1 million cells using Trizol (GibcoBRL) and was subsequently treated with DNAse (Boehringer Mannheim). Two strategies for RT reaction, differing in

priming and enzyme system, were compared. For both methods first-strand cDNA was generated from 1 μg of total RNA. Method 1 has been described previously as being an efficient pdT priming method and utilizes 100 units of Superscript II reverse transcriptase (Invitrogen) and 125 pmol oligo-$dT_{14}VN$ primer with 25 pmol 18S antisense primer [11]. Method 2 is based on a novel enzyme system and gene-specific priming. The RT-reaction was carried out by using a combination of 11 pmol 18S antisense primer with 40 pmol of gene specific RT-primers directed to different genes (Table 1) and 134 units of Superscript III RT (Invitrogen) in a total volume of 25 µl at 50 ºC for one hour, while keeping buffer conditions similar as described for method 1. The reaction mix was heated to 70 ºC for 15 min and then cooled to 4 ºC. The resulting gene-specific RT reaction mixes were column purified to remove gene-specific primers using Qiaquick PCR Purification Kit (Qiagen). The final volumes of the RT reaction mixes from both methods were 50 µl.

Amplification of cDNA fragments was performed in real-time PCR in a total volume of 10 µl using 1 µl of RT reaction mix and LightCycler FastStart DNA Master SYBR® Green I reagent (Roche, Basel, Switzerland) according to manufacturers' instructions. Primers and reaction conditions used are depicted in Table 1. The thermal cycling profile of the touchdown PCR was as follows: denaturation at 94 °C for 10 min, followed by 40 cycles of 94 °C for 1 s, primer annealing at 68°C for 7 s with a 0.5 °C/cycle decrease until 63°C, and extension for 40 s at 72 °C. Fluorescent data were acquired at the end of each extension phase. To determine that fluorescence was due to the amplification of a single PCR fragment, a melting curve analysis and agarose gel electrophoresis was performed. Five independent RT-PCR reactions per sample were analyzed using mastermixes for both the RT and PCR reaction. To determine the presence of contaminating genomic DNA in the RNA samples, cDNA synthesis reactions were performed in absence of reverse transcriptase and then served as template in PCR. No product was observed (data not shown). The mRNA quantification was carried out by analyzing linear regression on the Log (fluorescence) per cycle number data using the LinRegPCR programme [15]. For each sample the mRNA starting level was normalized for the 18S rRNA starting level. The variability of RT-PCR reactions between the five replicate samples was expressed as the coefficient of variation (CV) of the normalized mRNA starting levels that were additionally standardized for average mRNA starting levels of the two liver samples to correct for variations between PCR runs. By using different amounts of total RNA, we established a linear relation between the amount of total RNA added and the estimated mRNA starting level after analysis of real-time RT-PCR data (data not shown).

Fig. 1 shows the relation between the mean mRNA starting levels normalized for 18S rRNA and the CV obtained with the two RT methods. The scatter plots confirm that real-time

RT-PCR analyses of low expressed genes are less reliable than the data of high expressed genes. The high CV values could be solely attributed to variation in the RT reaction, since RT-PCR runs from duplicate cDNA samples generated less than 4% variability (data not shown). Using SPSS 10.0 software, the relation between the logarithm of the relative mRNA starting level and the CV is best described as linear using the data set obtained with method 1 (R^2=0,60) and exponential using the data set obtained with method 2 (R^2=0,80). The observation that at high template concentrations most improvement of reproducibility was achieved by applying method 2 is also reflected by the finding that the mean CV of both sample sets, liver versus cBAL58, has significantly decreased by 71% and 35%, respectively, as compared to method 1 ($p<0.05$ in a paired samples T-test). On the whole, the mean CV of the method 2 data set (58%$\pm$40 SD) is significantly lower than the mean CV (103%$\pm$50 SD) of the method 1 data set ($p<0.05$ in a paired samples T-test). The mean CV has decreased by 31% to 60% for the individual genes. Student T-tests showed that the estimated 18S normalized mRNA starting levels of GSTπ in the combined liver samples were different ($p<0.05$) between both methods, in contrast to all other gene/sample combinations tested. So, performing a real-time RT-PCR using method 1 may still generate reliable data, however these experiments should then be repeated four to five times, whereas method 2 experiments require less replicates, particularly for high template concentration samples. Together, these data show that method 2 clearly and consistently improves the reproducibility of the real-time RT-PCR for samples that vary in their expression levels of target genes over a broad range. To determine separately the contribution of the reverse transcriptase and priming strategy to the improvement in reproducibility, we varied the RT strategies and compared the CV of RT-PCR data averaged for four independent RT-PCRs of four different gene/sample combinations: AAT/liver, albumin/cBAL58, GSTπ/liver and transferrin/liver. In addition we used similar amounts of reverse transcriptase (134 units/ RT reaction). Although statistical analysis (Anova) showed no significant differences in CV between the four RT-strategies, some trends were observed. The average CV of the Superscript II/pdT group (89%$\pm$79 SD) was two-fold higher as compared to the Superscript III/gene-specific group (47%$\pm$26 SD). Since the average CV of both hybrid strategies, Superscript II/gene-specific and Superscript III/pdT were 54%$\pm$41 SD and 83%$\pm$79 SD, respectively, it seemed that gene-specific priming contributed 5-6 fold more to improvement of the reproducibility of the RT-PCR than the RT used. Superscript III is an MMLV reverse transcriptase with increased thermostability and half-life and with reduced RNase H activity rendering an increased activity. In addition, gene-specific priming is thought to increase the gene-specific template concentration. Thus the RT reaction is less susceptible to experimental variation by an increase in the activity as well as the specificity of the RT reaction. To successfully apply method 2 some aspects should be emphasized. Firstly, the

***Table 1**. Primers and conditions used in RT-PCR analysis*

Gene	primers 5' → 3'		Amplicon size (bp)	Real-time RT-PCR conditions		
				[$MgCl_2$] (mM)	Template dilution	Annealing temp. (°C)
18S rRNA	RT S AS	CGAACCTCCGACTTTCGTTT TTCGGAACTGAGGCCATGAT CGAACCTCCGACTTTCGTTCT	151	3	1000 X	68 ↓ 63
AAT	RT S AS	GGGGGATAGACATGGGTATGG ACAGAAGGTCTGCCAGCTTC GATGGTCAGCACAGCCTTAT	181	4		68 ↓ 63
Albumin	RT S AS	ACTTCCAGAGCTGAAAAGCATGGTC TGAGCAGCTTGGAGAGTACA GTTCAGGACCACGGATAGAT	189	4		68 ↓ 63
GST π	RT S AS	AGCAGGTCCAGCAG GTTG GCCAGAGCTGGAAGGAGG TTCTGGGACAGCAGGGTC	333	5	10 X	70 ↓ 63
Transferrin	RT S AS	CCAGACCACACTTGCCCGCTATG GAAGGACCTGCTGTTTAAGG CTCCATCCAAGCTCATGGC	310	2		68 ↓ 63

distance between the target sequences of the RT primer and the antisense PCR primer should be as small as possible to minimize the occurrence of truncated cDNAs not covering the whole amplicon. In the current experiment the distances were 26 bp (AAT) to 65 bp (albumin). Secondly, the temperature of the RT reaction in method 2 is 50ºC instead of 42ºC. This may inhibit secondary structure formation of the mRNA, which is a major obstacle in the preparation of high quality cDNA. Finally, the removal of the RT primer after completion of the RT reaction is essential to achieve specific PCR, without any interference of the RT primer remaining in the cDNA preparation. The purification of method 1 RT reactions did not affect the outcomes of the real-time RT-PCR. This may be explained by low availability of the pdT primer which anneals to all cDNAs generated from expressed RNAs, whereas the gene-specific RT primer is annealing only to the target cDNA, thereby drastically increasing the chance that the RT primer will function as antisense primer in the PCR reaction.

To our view the major limitation of method 2 is that the target genes need to be defined prior to the RT reaction and therefore method 2 is less flexible. Yet, the RT primers directed to different genes can be easily combined, up to at least eight primers, in a single RT reaction. In conclusion, method 2 will yield a marked increase in reproducibility in RT-PCR analyses of high and medium template samples. However, the reproducibility of the real-time RT-PCR of samples with low template concentrations is still relatively low, so multiple replicates need to be tested for accurate quantification of these samples.

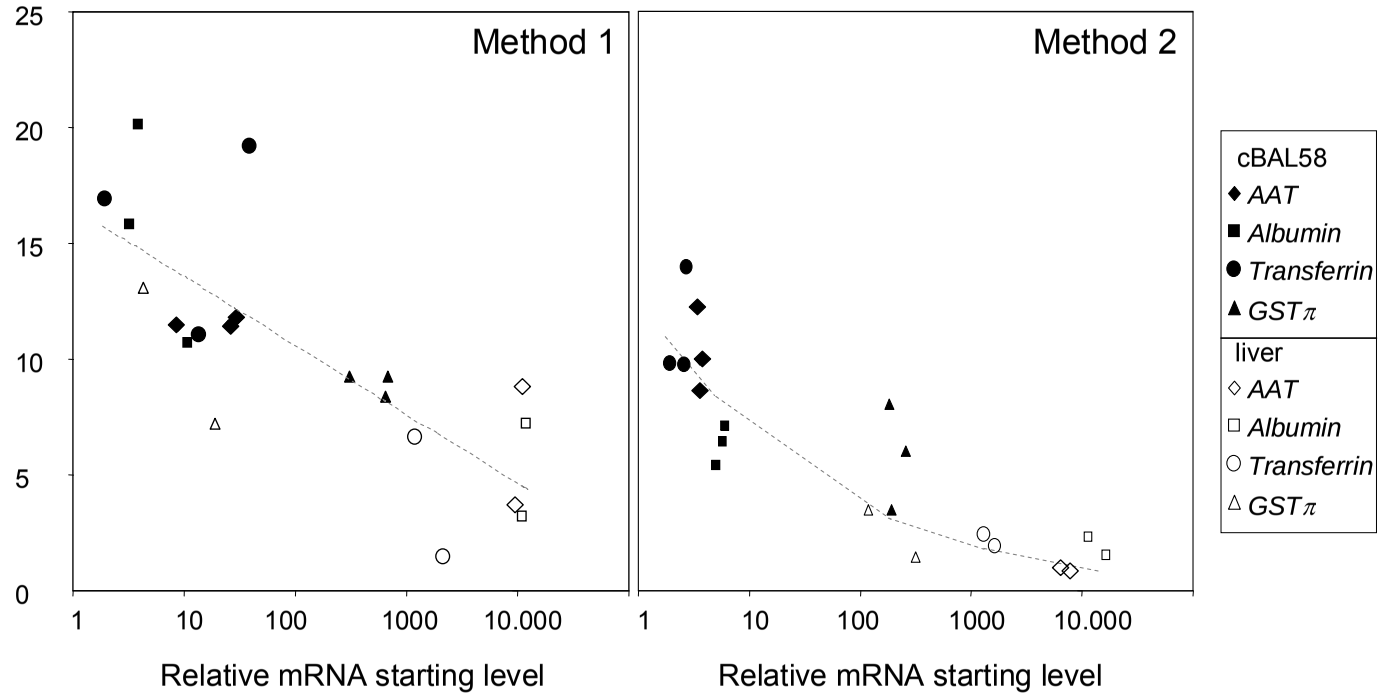

***Figure 1**: The relative mRNA starting level and CV of real-time RT-PCR reactions for different genes using liver (open symbols) and cBAL58 (closed symbols) samples using the two methods as indicated (n=5). A dashed line represents the best-fitted curve.*

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Chapter 7

Summary and general discussion

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Summary

Chapter 1, the introduction of this thesis about the development of a human hepatocyte cell line for the use in the AMC bioartificial liver (BAL), describes the difficulties encountered in defining the functional demands for an effective BAL treatment of acute liver failure (ALF), due to the high variability in etiology and pathogenesis of ALF and the lack in knowledge of the relevant molecular mechanisms. BAL systems based on freshly isolated porcine hepatocytes prolonged the life span in ALF animal models, but xenotransplantation-related disadvantages of porcine cells urges the development of a human cell source. To date, human hepatocyte cell lines obtained by *in vitro* immortalisation are most promising as future cell source for BAL application considering availability, functionality and safety. The introduction summarizes the characteristics of most important human hepatocyte cell lines currently available. In addition, this chapter emphasizes the importance of including freshly isolated mature hepatocytes when analysing hepatic functions of alternative cell sources.

One of the most promising human hepatocyte cell lines, the NKNT-3 cell line, was introduced by Kobayashi et al. in 2000 (1). The proliferative capacity of this cell line can be repressed by deletion of the immortalising gene, which stimulates hepatic differentiation of the cell line. This cell line showed full hepatic functionality, when transplanted into the spleen of 90% hepatectomized rats. We investigated the *in vitro* functionality of NKNT-3 cells and compared this functionality with that of freshly isolated human hepatocytes. The results, described in **Chapter 2**, showed that although inhibition of proliferation stimulated differentiation, the *in vitro* functionality of NKNT-3 cells was insufficient for BAL application, and forced us to explore other cell sources.

In **Chapter 3** the possibility of using human fetal liver cells (HFLCs) as a cell source for BAL application was investigated. In contrast to mature human hepatocytes, HFLCs are able to perform up to 58 cell divisions *in vitro*, which makes HFLCs an attractive cell source for BAL applications. Freshly isolated HFLCs exhibited albumin production and mRNA levels of albumin, transferrin, α-1-antitrypsin, hepatocyte nuclear factor 1α and 4 comparable to that of primary mature human hepatocytes, but more complex functions, like expression of cytochrome p450 and urea production from ammonia, were lower in HFLCs when compared to mature hepatocytes. Unfortunately, when HFLCs were expanded *in vitro*, the liver-specific functionality rapidly diminished. This process could partly be prevented by selecting functional clonal derivatives from the HFLC cell population. However, although the functionality of some clonal derivatives was higher than the functionality of *in vitro* expanded HFLCs, it was still not sufficient for BAL application.

The HFLCs as well as the clonal derivatives showed limited life spans. In one of the clonal derivatives, the growth arrest was overcome by immortalisation which is described in **Chapter 4**. The overexpression of telomerase reverse transcriptase immortalised clonal derivative cBAL08 and resulted in the novel human hepatocyte cell line cBAL111. The immortalisation process had not altered the majority of functional characteristics; cBAL08 and cBAL111 were comparable in their urea production rate and the hepatocyte specific mRNA levels. To investigate whether cell line cBAL111 was able to reach full hepatic differentiation, it was marked with green fluorescence protein (GFP) and transplanted into the spleen of immunodeficient mice. The GFP marked cBAL111 cells engrafted in the liver and a small part of these cells differentiated into mature human hepatocytes, without any indication of cell fusion between the murine hepatocytes and the cBAL111 cells. These results show that cBAL111 cell are able to full hepatic differentiation when exposed to the right stimuli, which in the transplantation experiment were provided by the liver environment.

The next challenge was to mimic *in vitro* the stimuli that made cBAL111 cells differentiate into mature hepatocytes *in vivo*. In **Chapter 5** the effects of culture time on the *in vitro* functionality were investigated. The urea production rate and galactose elimination rate of cBAL111 increased between 2 and 15 days culture time and remained constant from then on. By microarray analysis we tried to identify the underlying regulatory pathways. As expected, most genes and pathways that showed to be differentially expressed in this analysis were involved in replication and proliferation processes. Unfortunately, no clues for optimal hepatic differentiation were identified in this analysis.

Part of the conclusions described in this thesis is based on the determination of mRNA levels of hepatic genes. Initially, the technique of quantitative real-time RT-PCR that was used to measure mRNA levels, showed high variability between replicate samples. Therefore the technique of reverse transcription was investigated for samples differing to large extent in their mRNA levels of specific genes and was subsequently improved by gene-specific priming of the reverse transcription step and using an improved reverse transcriptase. This technique is described in **Chapter 6**.

General discussion

The effort that has been put in the development of *in vitro* hepatic functionality, resulted in many different human hepatic cell lines, but none of them performs the full hepatic function at the level of freshly isolated mature hepatocytes, the only cell source that gives BAL systems life supporting capacity so far.

We developed a new human hepatocyte cell line, cBAL111, a human fetal liver cell line immortalised by the overexpression of the human telomerase reverse transcriptase. This cell line might be an interesting addition to the already existing cell lines because it performs

all of the hepatic functions we investigated so far, although not to the extent of freshly isolated mature human hepatocytes. Increasing the level of hepatic differentiation is the next challenge in this field of research. Unravelling the stimuli that cells need to adapt hepatocyte fate *in vivo* is necessary to direct hepatic differentiation *in vitro*. Genome wide comparison of expression levels between primary mature hepatocytes and alternative cell sources, before and after *in vivo* differentiation, might identify crucial signalling pathways and stimuli.

The level of hepatic functionality can possibly be induced, *e.g.* by improving culture conditions to mimic the *in vivo* hepatocyte environment. In this respect, attention should be paid to the medium composition, especially to serum percentage, the composition of the exctracellular matrix and cell-cell interactions both between hepatocytes as well as between hepatocytes and non-parenchymal liver cells.

In addition to changing culture conditions, overall hepatic differentiation can also be stimulated by genetic modification, like the overexpression of liver enriched transcription factors *e.g.* HNF4 (2) or overexpression of cell cycle inhibitors like p21, which induce differentiation as well as proliferation inhibition (3). On the other hand, specific hepatic functions, known to be crucial for supporting patients with ALF, can be induced into cell lines by overexpression of rate limiting enzymes. As an example, in HepG2 cells overexpression of glutamine synthetase (GS) (4), urea cycle enzymes ornithine transcarbamoylase and arginase I (5) or cytochrome P450 3A4 (6), led to ammonia elimination instead of production, urea production or diazepam elimination, respectively. Alternatively, cell lines can be protected against apoptosis signals in the plasma of an ALF patient by introduction of Bcl-2 (7).

It cannot be excluded that a single cell line cannot display the full window of hepatic functions, even under optimal conditions. Even in the human liver, hepatocyte functionality is characterized by zonal differences. *In vivo*, pericentral cells are, for example, the only ones that express GS in contrast to periportal and mid-zonal hepatocytes (8; 9). To obtain liver functionality as completely as possible it might be necessary to combine two or more liver cell lines in one BAL, or to combine two or more BALs with different differentiation status characteristic for specific zones in the liver.

When more information about the molecular mechanism of hepatic encephalopathy and the needs of the different groups of ALF patients become available, it might even be possible to produce patient-tailored BAL systems (10). In the future it may be possible to characterise each patient based on the proteomic profile of the patient's blood or of a liver biopsy. Based on this information, cell lines and differentiation strategies of those cell lines can be selected that will optimally help the patients.

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Chapter 8

Nederlandse samenvatting

Nederlandse samenvatting en discussie

Samenvatting

In **Hoofdstuk 1**, de inleiding van dit proefschrift over de ontwikkeling van een humane levercellijn voor de AMC bio-artificiële lever (BAL), wordt ingegaan op het vraagstuk aan welke functionele eisen een BAL systeem moet voldoen om een effectieve behandeling van acuut leverfalen (ALF) mogelijk te maken. Met name het gebrek aan kennis over de moleculaire mechanismen en de variatie in etiologie en pathogenese van ALF bemoeilijken het ontwikkelen van nieuwe BAL therapieën. BAL systemen gebaseerd op vers geïsoleerde varkenshepatocyten verlengen de levensduur in diermodellen van ALF, maar xenotransplantatie-gerelateerde problemen maken de ontwikkeling van humane cellen noodzakelijk. Beschikbaarheid, functie en veiligheidsaspecten in ogenschouw nemend, zijn humane hepatocyt cellijnen, verkregen door *in vitro* immortalisatie, op dit moment de meest kansrijke optie voor toekomstige BAL systemen. In de inleiding worden de eigenschappen van de belangrijkste hepatocyt cellijnen van dit moment besproken. Tevens wordt in dit hoofdstuk benadrukt dat vers geïsoleerde humane hepatocyten in geen enkele analyse van alternatieve cellijnen voor BAL applicaties mogen ontbreken.

In 2000 introduceerde Kobayashi et al. één van de meest interessante humane hepatocyt cellijnen, de NKNT-3 cellijn (1). Het proliferatie vermogen van deze cellijn kan worden onderdrukt door de verwijdering van het immortaliserende gen; dit proces stimuleert vervolgens de hepatische differentiatie van de cellijn. De cellijn werd getransplanteerd in de milt van ratten die een 90% hepatectomie hadden ondergaan en kon de leverfunctie van deze ratten vervangen. Wij hebben de *in vitro* functionaliteit van deze cellijn onderzocht en vergeleken met die van vers geïsoleerde humane hepatocyten. De resultaten van deze vergelijking zijn beschreven in **Hoofdstuk 2** en laten zien dat hepatische differentiatie inderdaad optreedt door het remmen van de proliferatie, echter het niveau van de leverfunctie was onvoldoende voor gebruik in een BAL systeem. Het ontwikkelen van een andere cellijn bleek hierdoor noodzakelijk.

In **Hoofdstuk 3** worden de mogelijkheden van humane foetale levercellen (HFLCs) als celmassa voor BAL toepassingen onderzocht. In tegenstelling tot volwassen humane levercellen, kunnen HFLCs tot 58 celdelingen *in vitro* doormaken. Dit maakt deze cellen tot een interessant celtype voor BAL toepassingen. Vers geïsoleerde HFLCs toonden albumine productie en mRNA niveaus van albumine, transferrine, α-1-antitrypsine, hepatocyte nuclear factor 1α en 4 die vergelijkbaar zijn met volwassen humane hepatocyten. Echter, complexere functies zoals cytochrome p450 expressie en ureum productie uit ammoniak waren lager dan die van volwassen humane hepatocyten. Daarbij komt dat de initiële functionaliteit van HFLCs snel verminderde wanneer de cellen *in vitro* prolifereerden. Dit proces kon voor een

deel worden voorkomen door celklonen met hoge functionaliteit te selecteren uit de initiële celpopulatie. Hiermee werden klonen verkregen die na proliferatie een hogere functionaliteit behielden dan de HFLCs. Wederom bleek echter ook van deze klonen de functionaliteit onvoldoende voor BAL toepassingen.

Zowel de HFLCs als de hieruit gekloneerde cellijnen hebben een beperkte proliferatie capaciteit. Eén van de cellijnen, cBAL08 kon echter geïmmortaliseerd worden door de overexpressie van humaan telomerase reverse transcriptase. Dit proces, beschreven in **Hoofdstuk 4,** resulteerde in de nieuwe humane hepatocyt cellijn cBAL111. De immortalisatie had geen grote veranderingen in de functionaliteit van de cellijn tot gevolg; cBAL08 en cBAL111 hadden vergelijkbare ureum productie niveaus en hepatocyt specifieke mRNA niveaus. In een transplantatie experiment werd het differentiatievermogen van cBAL111 bepaald; cBAL111 werd gelabeld met green fluorescence protein (GFP) en getransplanteerd in de milt van immunodeficiënte muizen. De GFP gelabelde cellen bereikten de lever en een klein aantal cellen differentieerde tot volwassen humane hepatocyten, zonder dat er sprake was van fusie met muis hepatocyten. Hieruit werd geconcludeerd dat cBAL111 cellen de capaciteit hebben om volledig tot hepatocyt te differentiëren, wanneer daarvoor de juiste stimuli aanwezig zijn. In het transplantatie experiment werd deze stimulatie waarschijnlijk veroorzaakt door naburige cellen en matrix van de muizenlever.

De volgende uitdaging was het *in vitro* nabootsen van de stimuli die in het transplantatie experiment hadden geleid tot hepatische differentiatie. In **Hoofdstuk 5** is het effect van kweekduur op de *in vitro* functionaliteit beschreven. Na een kweekduur van 15 dagen waren ureum productie en galactose eliminatie van cBAL111 verhoogd ten opzichte van 2 dagen kweekduur en bleven nadien constant. Door middel van microarray analyse, is geprobeerd de onderliggende regulatie op transcriptie niveau te bepalen. Zoals verwacht, zijn het voornamelijk replicatie en proliferatie gerelateerde genen die differentieel tot expressie komen in deze vergelijking. Helaas zijn er geen aanwijzingen voor de regulatie van optimale hepatische differentiatie gevonden in deze analyse.

Een deel van de resultaten beschreven in dit proefschrift zijn gebaseerd op de bepaling van mRNA niveaus van lever specifieke genen. Bij de start van onze analyses vertoonde de techniek waarmee deze mRNA niveaus werden bepaald, de zogenaamde kwantitatieve real-time RT-PCR, een grote variatie tussen duplicaat monsters. De reverse transcriptie techniek is daarom onderzocht en verbeterd door het gebruik van gen-specifieke primers en een verbeterd reverse transcriptase enzym. Dit wordt beschreven in **Hoofdstuk 6**.

Discussie

Diverse initiatieven die zijn genomen om *in vitro* leverfunctie te ontwikkelen, hebben verschillende humane hepatocyt cellijnen opgeleverd, maar geen enkele van deze cellijnen functioneert op het niveau van vers geïsoleerde volwassen humane hepatocyten. Vers geïsoleerde volwassen humane hepatocyten zijn op dit moment dan ook de enige cellen die BAL systemen een levensverlengende effectiviteit kunnen geven.

Wij hebben een nieuwe humane hepatocyt cellijn ontwikkeld; deze cellijn, cBAL111, is een humane foetale levercel die door de overexpressie van humaan telomerase reverse transcriptase geïmmortaliseerd is. Deze cellijn is een waardevolle toevoeging aan de reeds bestaande cellijnen, omdat cBAL111 alle tot zover onderzochte functies uitvoert, al is het niveau van de functies nog onvoldoende voor BAL applicaties. Het verbeteren van deze functies is dan ook de volgende uitdaging in dit onderzoek. Het identificeren van de factoren die *in vivo* differentiatie van hepatocyten sturen is van belang voor hepatische differentiatie *in vitro*. Wellicht dat een vergelijking van het transcriptoom van vers geïsoleerde volwassen humane hepatocyten en de nieuwe cellijnen, voor en na *in vivo* differentiatie, cruciale regulatie cascades identificeert.

Hepatische differentiatie kan mogelijk worden geïnduceerd door met kweekcondities de leveromgeving *in vivo* na te bootsen. Hierbij kan gedacht worden aan de samenstelling van het medium, met name het serum gehalte, de samenstelling van de extra-cellulaire matrix en cel-cel interacties met zowel hepatocyten als andere levercellen.

Naast stimuli in kweekcondities, kan algemene hepatische differentiatie ook worden gestimuleerd door genetische modificatie, zoals overexpressie van in lever verrijkte transcriptie factoren, zoals HNF4 (2) of overexpressie van cel cyclus inhibitors zoals p21, welke differentiatie induceren en proliferatie remmen (3). Aan de andere kant kunnen leverfuncties, waarvan bekend is dat zij cruciaal zijn voor het behandelen van ALF patiënten, specifiek geïnduceerd worden door de overexpressie van snelheidsbeperkende enzymen. Zo leidde de overexpressie van glutamine synthetase (GS)(4), de ureum cyclus enzymen ornithine transcarbamoylase en arginase I (5) of cytochrome P450 3A4 (6) tot respectievelijk een netto ammoniak eliminatie in plaats van productie, tot ureumproductie en diazepam eliminatie. Op een vergelijkbare manier kunnen cellijnen ook beschermd worden tegen externe apoptose signalen, die in het plasma van ALF patiënten kunnen voorkomen, door de introductie van Bcl-2 (7).

Het kan niet worden uitgesloten dat één enkele cellijn nooit de volledige leverfunctie kan uitvoeren, zelfs niet onder optimale condities. In de menselijke lever worden verschillende leverfuncties in verschillende zones uitgevoerd. *In vivo* zijn b.v. pericentrale cellen de enige cellen die GS tot expressie brengen, in tegenstelling tot periportale en mid-zonale hepatocyten (8; 9). Om de leverfunctie in zijn totaliteit na te bootsen *in vitro*, kan het

noodzakelijk zijn om meerdere cellijnen of meerdere BAL systemen met verschillende differentiatie condities te combineren.

Zodra meer kennis beschikbaar is over het moleculaire mechanisme dat ten grondslag ligt aan het multi-orgaan falen (inclusief de hepatische encephalopathie) als gevolg van ALF en over de specifieke pathofysiologische kenmerken van de verschillende groepen ALF patiënten, zou het zelfs mogelijk kunnen zijn dat een ALF patiënt een 'op maat gemaakte' BAL therapie ontvangt (10). Wellicht dat in de toekomst de ALF patiënt op basis van een eiwitprofiel van het bloed en/of een leverbiopsie gekarakteriseerd wordt en op basis daarvan een optimale kunstlever behandeling ontvangt, met voor hem of haar op maat gesneden biocomponent in de BAL.

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Dankwoord

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