Preclinical development of the AMC-HepaRG-Bioartificial Liver

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CHAPTER 2

Bioartificial livers in vitro and in vivo: tailoring biocomponents to the expanding variety of applications


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ABSTRACT

Introduction: Bioartificial livers (BALs) were originally developed to treat patients suffering from severe liver failure, and relied on primary hepatocytes or on hepatoblastoma derived cell lines. Currently, new in vitro BAL applications are emerging, including drug toxicity testing, disease modelling and basic clinical research, and in recent years, advances in the field of stem cell biology have resulted in potential alternative cell sources.

Methods: This review identifies the demands of clinical and in vitro BAL applications to their biocomponent and summarizes the functionality and developmental state of BAL technology and cell types currently available. Relevant studies identified by searching the MEDLINE database until April 2014 were reviewed.

Results and conclusions: BALs have the potential to meet demands currently left unmet in both clinical and in vitro applications. All the reviewed biocomponents show limitations towards one or more BAL applications. However, the generation of stem cell derived hepatocyte-like cells is progressing rapidly, so the criteria for patient-specific drug toxicity screening and disease modelling are probably met in the near future. HepaRG cells are the most promising biocomponent for clinical BAL application, based on their proliferative and differentiation capacity.
INTRODUCTION

Bioartificial livers (BALs) have originally been developed to treat patients suffering from acute liver failure (ALF) and acute-on-chronic liver failure (ACLF), both devastating syndromes with mortality rates of up to 80%[1]. A clinical BAL is a device that consists of a bioreactor filled with liver cells, the biocomponent, and is connected extracorporeally to the blood circulation of the patient. Clinical application is still a primary aim; clinical trials of several BAL systems are in preparation (AMC-BAL [2] and UCL Alginate encapsulated HepG2 BAL [3]) or recruiting at present (Extracorporeal Liver Assist Device, ELAD [4]).

BAL culture promotes hepatocyte differentiation, facilitates growth in 3D and sustains the differentiated hepatocyte phenotype over a longer time compared to monolayer cultures [2, 5]. In addition, perfused oxygenated systems can provide a high level of control over the cellular microenvironment and flexibility in cell-medium ratio, sampling volume and regime [5, 6]. For these reasons, new applications for BALs are emerging, including drug development and toxicity screening, disease modelling and fundamental (stem) cell biology. These applications call for a much smaller device than their clinical counterparts. This can be accomplished by downsizing existing designs, but progression in the field of microfluidics has made ‘chip-sized organs’, including livers, a possibility [6-8].

Traditionally, BALs relied on primary hepatocytes or hepatoblastoma cell lines. In recent years many advancements in the field of regenerative and stem-cell medicine have been reported, especially on hepatocyte-like cells derived from stem cells. Our group has previously published an overview of liver cell culture devices[9] and of proliferative human cell sources applied as biocomponent in BALs [10]. An in depth review of clinical BAL systems has been published by Park et al [11]. Now, in the light of the expansion of in vitro BAL applications and new cell sources, we aim to provide an overview of BAL applications, their demands to bioreactor design and cell functionality in the first part, and in the second part to what extent these demands are met by the cell types currently available.

For this review we defined BALs as follows: perfused devices, loaded with living cells that exhibit liver specific functionality. We performed a Medline search with terms related to BALs, hepatocytes and hepatocyte-like cells, and selected papers until April 2014 on the basis of relevance for our aims and availability of full text in the English language.

Clinical BAL application

Clinical BALs are the largest BAL devices, intended to support liver function of patients who suffer from ALF or ACLF, two related syndromes with variable causes and courses, both characterized by acute massive necrosis of the hepatic cell compartment, leading to loss
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of liver functionality, accumulation of (neuro)toxins and to a strong immune response [1]. In this review we will use the term Severe Liver Failure (SLF) to indicate both. The majority of ALF patients in historic cohorts died due to intracranial hypertension [12, 13], but with modern intensive care management in specialized units, the proportion of these patients has dropped to 22% [13, 14]. This suggests that, next to intracranial hypertension and hepatic encephalopathy, other SLF components, such as inflammation, should be therapeutic targets. It is well-documented that SLF is associated with high plasma levels of several pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα) and pro-inflammatory interleukins, which contribute to the development of systemic inflammatory response syndrome (SIRS), and eventually will lead to multi-organ failure and death [15]. In a series of 887 ALF patients, SIRS was diagnosed in 57% of cases, and was associated with more severe disease outcome and a higher mortality [16].

To date, the only available curative therapy for SLF is liver transplantation, either orthotopic, in which the native liver is removed, or auxiliary, when the native liver is left in situ at least partially. After auxiliary liver transplantation, two thirds of surviving ALF patients undergo regeneration of their native liver [17, 18], indicating that temporary liver support, such as BAL therapy, is potentially a curative treatment for the majority of SLF patients.

On the basis of data from animal studies, Tsiaoussis et al. calculated that the minimum amount of high quality primary hepatocytes for effective liver support is 20-40% of liver-cell mass, which translates to approximately 15·10⁹ cells or 150 grams of hepatocytes [19]. Data from a hepatocyte transplantation study in a partial liver ischaemia rat model of ALF suggest that a biomass as little as 2% of native liver mass can have a beneficial effect through disinhibition of liver regeneration; the treated rats showed a marked increase in liver regeneration and survival time, but not in overall survival. This suggests that the newly regenerated livers (30% of original liver cells mass) were not able to sufficiently support the animals [20]. This suggests that the newly formed liver mass has not yet matured enough to support the animals, and underlines the importance of sufficient biomass to support the native liver during regeneration.

Besides cell mass, the efficacy of BAL therapy will be determined by the bioreactor configuration, as discussed in section 4, and by the functionality of the applied cells. The complex and largely non-elucidated cascade of events, as well as the variable nature of SLF, make specification of demands to liver cell functionality of clinical BALs challenging. SLF is a black box syndrome that can, at present, only be treated with a black box cure. Therefore, the phenotype of a clinical BAL biocomponent should approximate that of the most important epithelial cells in the liver: the primary human hepatocyte (PHH). We reviewed PHH functions
and their reported relevance in the setting of SLF treatment in Table 1 as a basis to screen potential clinical BAL biocomponents.

**In vitro applications**

Downscaled BALs were -and are still- used as tools to develop clinical BALs [21-23]. In addition, in recent years new applications for small scale in vitro BALs have emerged, such as drug development, disease modelling and, to a smaller extent, basic scientific research [6, 7].

**Drug development**

The most relevant in vitro BAL application in drug development is screening for hepatotoxicity, which is the main cause of late-stage failure and withdrawal of drugs from the clinical development and after introduction on the market [24]. Animal studies and in vitro studies with human hepatocytes and liver cell lines have limited predictive value, as illustrated by the fact that 38–51% of drug induced liver injuries (DILI) in humans are not detected preclinically [24].

The likelihood of a DILI differs between individuals, a phenomenon that can be explained by the inter-individual difference in expression of detoxification enzymes and corresponding metabolite profile, as well as the susceptibility to damage inducing molecules. It is thought that DILIs can occur through three pathways: direct cytotoxicity, direct mitochondrial impairment or specific immune reaction, as reviewed by Russmann *et al* [25]. The occurrence of DILIs therefore, depends on the abundance and activity of proteins involved in drug detoxification, as well as the metabolic state of the cells, the mitochondrial activity and the host immune system.

A drug toxicity screening-BAL should therefore exhibit full phase1 and 2 detoxification functions, express the entire array of basolateral and apical transporters and, at the same time, have physiological energy metabolism. Furthermore, this BAL should show high viability, reproducibility and stability. An *in vitro* BAL from a single genetic background, exhibiting all of the properties listed above would be an extremely valuable tool. Preferably, however, these drug toxicity screening BALs should be supplied with biocomponents of diverse genetic backgrounds with corresponding phenotype, reflecting the variability within the normal population [5].

**Liver disease modelling**

For a number of human liver diseases, ranging from infectious to metabolic and auto-immune diseases, there are no suitable *in vitro* models. Here we will discuss three notable examples of fields that could benefit from an *in vitro* BAL system in the near future, namely: *Plasmodium vivax* malaria, viral hepatitis and metabolic diseases [45, 46]. *P. vivax* and *P. ovale* can reside quiescently in human hepatocytes, as hypnozoites. The mechanism of parasites reactivation
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Table 1. Important hepatocyte functions for clinical BAL-biocomponents.

<table>
<thead>
<tr>
<th>Function</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia detoxification</td>
<td>There is a causal relationship between hyperammonaemia and intracranial hypertension in ALF. In vivo ammonia elimination occurs via two major routes: irreversible fixation into urea and reversible incorporation into amino acids, most notably glutamine. The latter route is thought to function as a temporary ammonia sink during SLF as in various cell types glutamine breakdown by glutaminase yields ammonia again. The clinical BAL must have the capacity to eliminate ammonia to the same extent as PHHs, preferably through urea cycle activity.</td>
</tr>
<tr>
<td>Lactate elimination</td>
<td>Hepatocytes, like many cell types, abundantly express lactate dehydrogenase (LDH), the enzyme that catalyses the reaction from lactic acid to pyruvate and back. In contrast to most cell types, PHHs eliminate excess lactate through this pathway. Lactate can effectively cross the blood-brain barrier and recent literature suggests a causal relation between increased cerebral lactate levels and the occurrence of cerebral edema in a rat study. In ALF patients brain lactate concentration correlates closely with ICP, pointing out lactate elimination as a potentially important BAL function. However, it is also well-documented that lactate acts as a cerebral energy source under both physiological and pathophysiological conditions. Deeper insight is required before superphysiological lactate clearance can be a therapeutic objective. For clinical BAL application, lactate elimination up to the level of PHHs appears preferable over lactate production.</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>The liver produces a multitude of vital proteins, some of which may attenuate SLF. Albumin, clotting factors and apolipoprotein A1 (Apo-A1) are examples of such substances, although the first two can be administered exogenously in the ICU. Albumin is the most abundant plasma protein synthesized by the liver and acts as a carrier protein for soluble molecules. It is an antioxidant and an important determinant of blood-colloid osmotic pressure. Albumin has been suggested to modulate immune responses by buffering neutrophil derived reactive oxygen species. Studies in mice suggest that Apo-A1 can reduce inflammation by augmenting the effectiveness of the regulatory T-cell response. These proteins could, as such, play a role in SLF disease outcome. However there is no evidence that shortages of specific proteins contribute to SLF mortality under optimal ICU care. Protein synthesis should be a quality parameter for a clinical BAL, because the hepatocyte proteome is a biological aspect that remains largely non-elucidated and is therefore a prototypical “black box” property that BALs have been envisioned to accommodate.</td>
</tr>
<tr>
<td>Carbohydrate and lipid metabolism</td>
<td>In the clinical setting, plasma glucose supplementation is standard of care, therefore this is not an important therapeutic target on its own. Lipid metabolism is altered in SLF patients, high density lipoprotein (HDL) serum levels are decreased during SLF and reflect disease severity but are no independent risk factor for mortality. No specific part of carbohydrate or lipid metabolism other than lactate elimination has been described as a specific BAL target.</td>
</tr>
</tbody>
</table>
Xenobiotic detoxification

The xenobiotic detoxification system, also involved in metabolizing endobiotics, has been proposed to be an important function of BAL systems in clearing toxins that accumulate during ALF [39]. The system exists of phase 1 (e.g. cytochrome P450) and phase 2 metabolizing enzymes, as well as transport proteins. There is evidence that neurotoxins normally metabolized by the liver, such as neurosteroids, accumulate during ALF and synergistically contribute to the development of hepatic encephalopathy in rodents [40]. Very little is known, however, about the contribution of such toxins to the clinical outcome of human subjects and the impact of the xenobiotic detoxification system. One study covering the subject [39] reported a ~40% increase in survival time in a diazepam-induced ALF model in dogs when supported with a BAL-system filled with CYP3A4 and Glutamine Synthetase (GS) overexpressing HepG2 cells (HepG2-GS-Cyp3A4).

However, when the experiment was repeated several years later, the authors reported a comparable increase of survival in animals only treated with HepG2-GS loaded BALs [39, 41].

There are no data that xenobiotic detoxification-competence is a prerequisite for efficacious BAL therapy, but there is some evidence that toxic compounds may contribute to disease severity. Therefore a basal level of xenobiotic detoxification is desirable in a clinical BAL, for example the expression of several detoxification enzymes with a broad substrate range, such as CYP3A4, CYP2D6 and glycosyltransferases [42].

Immunomodulation

Immunomodulation is not a classic hepatic function, but is relevant to the treatment of SLF patients because SIRS is a determinant of disease outcome [16], and because an increased concentration of pro-inflammatory cytokines in the liver is associated with inhibition of regeneration [43].

The our knowledge there is only one experimental report on the support of liver regeneration by BAL-treatment. In a partial liver resection and liver-ischaemia-model in rat, PHH-BAL-support increased survival time and expression of liver-enriched and growth-induced transcription factors in the livers of treated animals. This led the authors to conclude that BAL-therapy has a positive effect on liver regeneration. Furthermore they reported a decrease in transforming growth factor β1 (TGFβ1) plasma levels and hypothesised this could be the mechanism of action [44]. TGFβ1 is a potent inhibitor of hepatocyte proliferation in vitro and in vivo, but hepatocytes are known to lose their TGFβ1 sensitivity during regeneration [43], indicating the importance of other mechanisms that are not yet fully understood.

Immunomodulation may be of added value for SLF treatment through attenuation of SIRS and in the stimulation of liver regeneration, but the limited information on the mechanisms does not allow a suggestion for critical parameters.
is largely unknown. Diagnosis and treatment options are therefore limited, hindering the recently reaffirmed objective to eradicate malaria worldwide of especially *P. vivax* [47]. *In vivo*, *P. vivax* hypnozoites can only be studied in non-human primates, presumably because the parasite machinery requires highly differentiated primate hepatocytes. Until recently, a suitable *in vitro* model was not available, as the required fresh primate liver material is not stable enough to support the life-cycle of *P. vivax* [45]. In a recent paper, Dembele *et al.* reported on an advance towards the successful long-term infection of primary macaque hepatocytes with *P. cynomolgi*, a recognized *P. vivax*-model capable of infecting both humans and macaques [48]. Primary macaque hepatocytes were co-cultured with the human hepatic progenitor cell line HepaRG to rapidly fill up any breaches in cell-cell contact and thereby attenuate hepatocyte dedifferentiation. This model supported the formation and maintenance of hypnozoites, with infection load decreasing gradually over the course of 40 days of culture. The authors state that limitations of their model include a limited cell mass and detrimental breach of cell-cell contact [48]. An *in vitro* BAL system would be a logical follow-up model, provided that it sustains a higher number of cells and aids cell-cell contact by the means of a 3D configuration. At present, there have been no reports published of cells other than primary primate hepatocytes that can facilitate *Plasmodium* infection, illustrating the inherent difficulty in specifying biocomponent demands to model diseases that are not fully understood.

In Hepatitis B and C virus research, *in vivo* models currently include chimpanzees and transgenic humanized immunodeficient mice. There is no established *in vitro* system available that supports the entire life cycle of the viruses [46, 49]. An *in vitro* BAL system can be of value to this field by offering a stable liver model, provided that the biocomponent applied in such BALs is susceptible to infection by these viruses and able to sustain the entire viral lifecycle (Table 2).

The modelling of metabolic diseases relies on the ability of a biocomponent to express the specific hepatic phenotype associated with a disease, which is often genetically predisposed. This implies that the biocomponents applied in these models must be derived from different (epi)genetic backgrounds while retaining their phenotype (Table 2).

**Basic research**

In basic research there are several applications for *in vitro* BAL devices, including the study of cell-cell interaction in a 3D setting, and the influence of perfusion rate, nutrients and oxygenation on hepatocyte differentiation [6, 7, 50]. Especially microfluidic *in vitro* BALs have the potential to become valuable tools to study hepatocyte differentiation because of the high level of control over the micro-environment they offer, including nutrient and oxygen gradients [6]. The demands to the applied cell type or types for these applications are dependent on the research question and will therefore not be extensively explored in this review.
Table 2. Different sizes of BALs, their applications and cell source requirements.

<table>
<thead>
<tr>
<th>BAL size</th>
<th>Biomass required</th>
<th>Design</th>
<th>Limitations</th>
<th>Applications</th>
<th>Biomass functional requirements</th>
<th>Current developmental stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical size</td>
<td>~1.5×10^10 cells (~150g) [19]</td>
<td>Radial flow - Hollow fibre - Direct perfusion - Suspension</td>
<td>- High operational costs due to large size. - Lower level of control of the cellular (micro) environment compared to chip sized BALs</td>
<td>Treatment of patients suffering from SLF</td>
<td>- Basal level xenobiotic detoxification - Protein synthesis - Urea cycle activity - Stability in plasma environment - Financial feasibility of large scale production</td>
<td>Up to stage 3 clinical trial (HepatAssist, AMC-BAL and ELAD) [4, 55]</td>
</tr>
<tr>
<td>Intermediate size</td>
<td>10^6-10^9 cells [56, 57]</td>
<td>Radial flow - Hollow fibre - Direct perfusion - Suspension - Microfluidic</td>
<td>Lower level of control of the cellular (micro) environment compared to chip sized BALs</td>
<td>Developmental tool for clinical BALs</td>
<td>To be used with the biomass applied in the clinical counterpart</td>
<td>In use</td>
</tr>
<tr>
<td>Chip size</td>
<td>10^4-10^6 cells [7, 51]</td>
<td>Microfluidic</td>
<td>Limited sample volume</td>
<td>Preclinical hepatotoxicity screening</td>
<td>- Balanced expression of xenobiotic detoxification proteins - High viability - Stable phenotype during long-term culture - Physiological energy metabolism and mitochondrial function - Diverse genetic backgrounds, preferably including donors who suffered from DILI.</td>
<td>Under development</td>
</tr>
<tr>
<td>Inherited disease modelling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Cells sourced from diverse genetic background and phenotype. - High hepatic differentiation grade</td>
<td>Under development</td>
</tr>
<tr>
<td>Infectious disease modelling</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>- Infectability with pathogen of interest - Generally a high differentiation grade.</td>
<td>Under development</td>
</tr>
<tr>
<td>Research requiring strict control of the cellular microenvironment such as (stem) cell differentiation, experiments that involve very costly compounds and high throughput applications, not requiring large sample volumes.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Dependent on the type of research.</td>
<td>First commercial models entered end-user beta test phase.</td>
</tr>
</tbody>
</table>

BAL: bioartificial liver; DILI: drug induced liver failure; SLF: severe liver failure
BAL hardware

BAL-bioreactor models vary to large extent in size and configuration. Roughly, the bioreactors can be divided into 3 groups on the basis of their size, from chip-sized in vitro BALs loaded with less than one million cells, via intermediate sized in vitro BALs to clinical size BALs supporting up to $10^{11}$ cells [19, 51] (Table 2).

The configuration of the bioreactor has a direct impact on the functionality of the cells cultured in it. Characteristics with a large impact on functionality include: 3D vs 2D configuration, extracellular matrix composition, oxygenation, shear stress and exchange of nutrients and metabolites between cells and culture medium. The configuration will determine the level of control over the microenvironment, scalability and financial feasibility, often conflicting with one another. Therefore, the configuration will influence the applicability of a certain bioreactor model to different fields of research, as summarized in Table 2.

Cells reach a higher differentiation grade when cultured in 3D compared to 2D, a phenomenon thought to be mediated by improved cell-cell interaction and direct activation of differentiation pathways [2, 52]. 3D configuration can be achieved through a 3D scaffold or the use of spheroids: cell-aggregates in a 3D sphere-configuration. Spheroids can be deployed in BAL systems based on suspension culture [53], nano-patterned microfluidic chips [7] or encapsulated in a supportive matrix such as a hydrogel [54]. 3D cell configuration has proven feasible in BAL bioreactors of all sizes [5, 7, 51] and for this reason, should be considered state of the art.

The precise role of oxygenation tension during the different stages of hepatocyte development is not known. Terminally differentiated hepatocytes, however, require high oxygen tensions. Therefore, the general consensus is that sufficient oxygenation is a main challenge when designing a BAL-bioreactor [55].

Clinical and intermediate size BAL hardware

Small pore capillaries are regularly used to compartmentalize cells, medium and/or gas [11]. This technology is easily scalable between intermediate and clinical size BALs, capable of supporting cell numbers in the $10^7$-$10^{11}$ range, but not less. A disadvantage of compartmentalization of medium and cells is the hindrance of nutrient and metabolite exchange, a problem solved in the AMC-BAL by growing cells in a non-woven polyester matrix in direct contact with the perfusate, whilst securing oxygenation at site through gas-capillaries [21]. Other currently used clinical BALs are based on suspension cultures for spheroids or hydrogel encapsulated cells [3, 53]. The different options for clinical and in vitro BAL-bioreactor configuration have been reviewed in more detail in articles by Park et al. [11] and Godoy et al. [5].
Bioartificial livers in vitro and in vivo

Chip sized BAL hardware
A highly controlled microenvironment and small cell numbers are feasible in microfluidic BAL systems. Much as in the physiological situation, medium perfusion and substrate availability can be precisely controlled and quantified, aiding reproducibility and allowing the set-up of high throughput systems. The microfluidic design comes at the expense, however, of limited sample volume and limited and costly scalability. Still there is a group pioneering larger scale microfluidic devices, showing proof of principle that the microfluidic approach could work for a clinical BAL [58]. There are several chip sized systems in advanced stages of commercialization [5, 59]. The state of the art of chip sized organs has been concisely described in a paper by van de Stolpe et al [8].

Primary hepatocytes
PHHs are generally regarded as the gold standard for clinical BAL application and drug toxicity testing, being the main epithelial cell type responsible for the functions of interest in the physiological situation [5, 19]. There are, however, two major drawbacks to the use of primary hepatocytes for all BAL applications: availability and phenotypic instability.

Availability
Since PHHs undergo maximally 1-2 population doublings in vitro, and human livers are scarce, PHH's are not available in large numbers and batch sizes are limited. Although the availability of cells from diverse genetic backgrounds is favourable for many applications, for the reproducibility of both clinical and in vitro applications the limitation of hepatocyte batch size poses a challenge.

Xenogeneic primary hepatocytes have been suggested and applied as an alternative source of biomass for clinical BAL systems [11, 19] as well as for in vitro applications. Concerns about zoonosis have led to extremely strict regulations on the clinical application of xenogeneic material, de facto ruling out animal hepatocytes as a clinical BAL biocomponent. Xenogeneic primary hepatocytes also differ phenotypically from PHHs, resulting in different susceptibility to pathogens, drug metabolism and transporter activity, and are therefore regarded as unsuitable for many in vitro applications [48, 49, 60, 61].

PHHs can proliferate in vivo and repopulate animal livers. By transplantation of PHHs in immunodeficient mice, human hepatocytes are commercially produced [62]. Theoretically this technique could be expanded by using large animals. However, this does not solve the issues of zoonosis and phenotypic instability.

In vitro transduction with pro-proliferative genes to obtain expandable hepatocytes is propagated by Burkard et al [63]. PHHs transduced with a set of non-disclosed proliferation
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inducing genes’ are commercially available. However, their functionality does not approach that of PHHs in xenobiotic drug metabolism or ammonia detoxification [63].

Phenotypic instability
Hepatocytes are most commonly isolated through 2 step collagenase perfusion as first described by Seglen et al. in 1976 [64], which involves perfusion of liver tissue with a Ca\(^{2+}\) free medium to disrupt adhesion molecules and collagenase to free the hepatocytes from the extracellular matrix. Directly after isolation, hepatocytes start to dedifferentiate. Dedifferentiation is now thought to be an active process, rather than a gradual loss of cell viability, and is characterized by the upregulation of structural- and extracellular matrix proteins and an activation of a mechanism similar to epithelial-to-mesenchymal transition, driven by MAP-kinase and AKT signalling, leading to the loss of liver enriched transcription factors (LETFs) expression [65]. Over the course of as little as 24 hours, hepatocytes loose the majority of LETFs transcription, leading to a rapid decrease in transcription and synthesis of hepatic proteins. Several phase 1 and 2 detoxification enzymes and anti-oxidative proteins are amongst the most rapidly down-regulated proteins [60, 61]. A review covering strategies to counter hepatocyte dedifferentiation through interventions in cell-cell contact, paracrine signals, cell- extracellular matrix interaction, epigenetics, gene transcriptional and post-transcriptional processes was written by Fraczek et al. [65].

Regulatory authorities regard data gathered from fresh and cryopreserved PHHs to be of equal value [66], and thus these are frequently used as gold standard control in papers describing PHH alternatives. One should keep in mind, however, that reference PHHs constituting of cryopreserved PHHs or of PHHs at more than 1-2 days after isolation have a lower functionality compared to that of freshly isolated PHHs, leading to a major overestimation of the tested cell functionality.

Hepatocyte cell lines
The main advantages of hepatocyte cell lines are their almost unlimited proliferative capacity and the relatively cheap culture process. There are two general approaches to acquire proliferative hepatocyte cell lines: through genetic engineering of PHHs, or by isolating cells from liver tumours that have already acquired the necessary mutations to render the cells immortal [67]. Most of the available cell lines only exhibit marginal hepatocyte specific functionality, which can improve to some extent when cultured in a BAL [2, 5]. We and others have published extensive reviews on immortalized cell lines for application in clinical BAL and toxicology systems [10, 19, 68, 69]. Briefly, the most differentiated and most frequently applied cell lines are the hepatoblastoma derived HepG2 -especially its subclone HepG2/C3A- and the hepatocellular carcinoma derived progenitor cell line HepaRG.
HepG2 cells are robust and proliferate quickly, making them easy and economical to expand and to select or engineer sub-clones. Especially sub-clone HepG2/C3A exhibits some liver-specific functionality, most notably synthesis of albumin and low expression -but not inducibility- of several major CYPs. Carbohydrate metabolism and ammonia detoxification are not comparable to that of PHHs at all, since they do not detoxify ammonia and produce large amounts of lactate [27, 67, 70]. Some of the genes necessary for clinical BAL application have been introduced into HepG2 cells, including CYP3A4 and glutamine synthetase, to improve xenobiotic metabolism and ammonia elimination by glutamine synthesis, respectively. As discussed in Table 1, these cells have produced encouraging results in animal models of ALF and there is a theoretical ground to believe that treatment of SLF-cases with a pronounced hyperammonaemia component can be beneficial, but none of the HepG2-derived cells meet the clinical BAL criteria summarized in Table 1.

HepaRG is a bipotent hepatic progenitor cell line that differentiates into a mixed culture of hepatocyte- and cholangiocyte-like cells, and is generally regarded to be the most differentiated proliferation-competent alternative for PHHs currently available [10, 67]. HepaRG exhibit substantial phase1- and 2 drug metabolism, ammonia elimination, contact-inhibition, protein synthesis and lactate consumption when cultured in 3D [2]. A disadvantage of HepaRG is the slow growth rate: cells can be split in a 1:5 ratio only once every 2 weeks, and maximum differentiation is reached after approximately 4 weeks, making expansion time-consuming and relatively costly. Also, in our experience, the cells have the tendency to lose their hepatocyte phenotype as the passage number increases or if the culture protocol is slightly violated (unpublished data). HepaRG is the only cell line with the intrinsic ability to support the entire hepatitis B life cycle, indicating that this cell line is appropriate for infectious liver disease modelling.

In conclusion, the HepaRG line is superior to HepG2 in terms of hepatic functionality, but not in flexibility and costs. HepaRG meets the criteria for clinical and most in vitro BAL applications, while HepG2 only meets part of the criteria for clinical BAL application after genetic manipulation. A major disadvantage inherent to all cell lines is that they only represent the phenotype of one single donor.

**Stem cell derived hepatocytes**

Both pluripotent and multipotent stem cells can be directed towards differentiation into hepatocytes in vitro by defined chemical signals and/or by transduction with specific transcription factors. As of yet, nobody has reported the successful differentiation of stem cells into fully mature hepatocytes, therefore stem cell derived cells with hepatocyte characteristics are commonly referred to as hepatocyte-like cells (HLCs) [71]. Stem cells and derived HLCs require growth factor- and small molecule enriched culture media, and often
extracellular matrix substrate coating. These requirements make stem cells too costly to be applied in clinical BALs in the near future. The available human stem cells include: human adult stem cells, induced pluripotent stem cells (iPSC) and embryonic stem cells.

**Human adult stem cells**

In adults, there are several sources of stem cells that can potentially be harvested to create HLCs, including mesenchymal stem cells and human liver stem cells. Several reports have been published on mesenchymal stem cell-derived HLCs, although, as of yet, no evidence of a high differentiation grade or a comparison with PHHs has been provided [72, 73].

The existence of the resident human liver stem cell or progenitor cell remains the subject of debate, but several reports have been published on the isolation of cells from the human liver that exhibit proliferative capacity and the ability to differentiate into HLCs [74-76]. Fonsato et al. isolated human liver cells, named ‘human liver stem cells’, which stained positive for embryonic stem cell- and mesenchymal markers, and negative for hematopoietic markers [76]. When cultured in a BAL system, hepatic differentiation was induced. Functions exhibited by these cells include activity of several major CYPs, uptake and excretion of indocyanine green and production of albumin and urea. No further analyses of carbohydrate metabolism, ammonia detoxification or CYP inducibility were published.

Duret et al. have isolated nonparenchymal epithelial cells from liver tissue of patients who underwent a partial liver resection and proposed these to be the human liver progenitor cells [75]. These cells were negative for most classic hepatocyte- and stem cell markers, but had a high proliferation potential and could be differentiated into HLCs under the influence of the growth factors HGF, EGF and FGF. Liver specific gene transcription was induced, as well as production of albumin and alpha 1-antitrypsin. No comparison with PHHs or further functional characterisation was included.

**Induced pluripotent stem cells.**

In 2007 Takahashi et al. succeeded in generating pluripotent stem cells from human dermal fibroblasts by overexpressing four transcription factors (Oct 3/4, Sox2, c-Myc, and Klf4) [77]. This ground breaking work has led to a rapidly developing field with private and public hiPSC banks emerging worldwide, aiming to produce thousands of human derived cell lines over the coming years from donors with different genetic backgrounds, both healthy and diseased [78]. Thus, theoretically providing the ideal source of BAL biocomponents if iPSCs can be efficiently differentiated into functional hepatocytes.

iPSCs can be differentiated into HLCs in a heterogeneous culture with different grades of differentiation [79]. IPS-HLCs are capable of supporting the entire life cycle of the hepatitis C
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virus [80], and iPS-HLCs derived from subjects suffering from alpha 1-antitrypsin deficiency, familial hypercholesterolemia or glycogen storage disease type 1a showed preservation of the donors’ disease phenotype [81].

We have summarized the papers that report on the generation of HLCs from human iPSCs and that included at least functional data and a PHH control group in Table 3. Interpretation of the reported functionality is, however, often hampered by the limited or lacking information on PHH source and culture procedure, therefore the reported data may be significantly overestimated. Also, the available literature focusses on HLCs cultured in monolayer, but there are reports available indicating that HLC differentiation is promoted by 3D configuration [52, 82]. Activity and inducibility of major CYPs are reported which implicates usefulness to drug toxicity and -metabolism studies. However, variation in expression between the drug detoxification genes and transporters in combination with the mixed cell phenotype could impair their applicability. No information on respiratory status or mitochondrial function are available. Only one study reported on urea cycle activity as measured by heavy ammonia conversion, in which case no urea cycle activity was found [83]. All iPSC-HLCs expressed the foetal marker alpha-fetoprotein (AFP), although, noticeably, the co-cultured liver organoids of Takebe et al. expressed AFP only in 50% of the albumin positive cells [84]. AFP expression indicates that iPSC derived HLCs are of an immature hepatocyte phenotype, or in fact represent foetal liver cells rather than hepatocytes as proposed by Schwartz et al. and Hannan et al. [79, 94]. The authors propose this to be due to our lack of understanding of the physiological process of fetal liver maturation [79]. Takayama et al. and Yu et al. conclude that a better understanding of the physiological hepatic microenvironment and differentiation stimuli is required to produce cells with a higher differentiation grade [83, 89]. According to our best knowledge, confirmed by others [71], no data have been published on the long term phenotypic stability of stem cell derived HLCs in vitro, information that is necessary to assess the applicability of iPS-HLC in disease modelling and drug development.

Lineage reprogramming

Lineage reprogramming is an approach adapted from iPSC technology; a selected set of transcription factors is introduced into primary cells to directly reprogram them into a specific cell type, bypassing the state of pluripotency. Zhu et al. [91] directly reprogrammed fibroblasts into a multipotent state, comparable to endoderm, and subsequently differentiated the cells towards HLCs. These cells did not resemble terminally differentiated hepatocytes in their expression pattern of 1299 genes, and showed a very limited capacity to repopulate mouse livers compared to PHHs. The expression patterns of the cells changed drastically into that of PHHs after transplantation into mice, showing that the cells have the potency to fully differentiate under the right conditions (in vivo) into hepatocytes, but that, again, our knowledge to establish those conditions in vitro is still insufficient.
## Table 3. Summary of generated IPSs and lineage reprogramming-derived HLCs.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Efficiency</th>
<th>Hepatic functionality</th>
<th>Control PHHs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Song 2009 [85]</td>
<td>-iPSCs, chemically mediated differentiation -Mitomycin-C-treated feeder layer*</td>
<td>60% Albumin positive cells</td>
<td>-AFP positive -Urea synthesis ~10% of PHH -Albumin synthesis ~10% of PHH -Glycogen positive, PAS (periodic acid shift) staining -CYP3A4 activity ~3% of PHH</td>
<td>Cryopreserved PHHs from discarded donor organs, cultured for several days, AFP positive</td>
</tr>
<tr>
<td>Si-Tayeb 2010 [86]</td>
<td>-iPSCs, chemically mediated differentiation -Matrigel coated plates</td>
<td>~80% Albumin positive cells</td>
<td>-AFP positive -Urea synthesis =equal to HepG2 -ICG uptake and excretion -LDL accumulation -Glycogen positive, PAS staining -Lipid droplets uptake -Transcript levels CYPs &lt;1%</td>
<td>Human liver tissue for normalization of assayed transcript levels, source not discussed</td>
</tr>
<tr>
<td>Jozefczuk 2011 [87]</td>
<td>-iPSCs, 3-step cytokine driven differentiation -Matrigel coated plates</td>
<td>Heterologous population</td>
<td>-AFP positive -Urea synthesis ~80% of PHH -ICG uptake and excretion -Glycogen positive, PAS staining -Low CYP transcript levels</td>
<td>Commercial fresh PHHs, 1 donor, culture method not discussed, AFP negative</td>
</tr>
<tr>
<td>Chen 2012 [88]</td>
<td>-iPSCs, 3-step cytokine/ growth factor driven differentiation -Matrigel coated plates</td>
<td>70% cells positive for FOXA2 and Sox17</td>
<td>-AFP positive -Urea synthesis ~100% of PHH -CYP3A4 activity ~100% of PHH -Glycogen positive, PAS staining -HNF4a expression HSC&gt;PHH</td>
<td>Age, source and culture conditions PHHs not specified, AFP positivity suggesting low quality</td>
</tr>
<tr>
<td>Yu 2012 [83]</td>
<td>-iPSCs, 3-step cytokine/ growth factor driven differentiation -Matrigel coated plates</td>
<td>80% Albumin positive cells</td>
<td>-AFP positive -Urea synthesis ~25% of PHH, 0% -Conversion of heavy ammonia into urea (41.7% in PHH) -Albumin production ~25% of PHH -Diazepam hydroxylation ~30% of PHH (CYP3A4/2C19 activity) -Glycogen positive, PAS staining</td>
<td>Fresh PHHs, expanded in repopulated mice livers, AFP negative</td>
</tr>
<tr>
<td>Takayama 2012 (1) [89]</td>
<td>-iPSCs, differentiated in 3 stages, driven by growth factors cytokines and transduction with SOX17, HEX and HNF4α consecutively -Matrigel coated plates</td>
<td>~80% E-cadherin positive cells</td>
<td>-AFP positive -Sensitive to hepatotoxic compounds -Expression and inducibility of several major CYPs -Glycogen positive, PAS staining -ICG uptake and excretion</td>
<td>Cryopreserved PHHs, single donor, tested 48 hr after plating on collagen coated plates, AFP negative</td>
</tr>
<tr>
<td>Takayama 2012 (2) [90]</td>
<td>-iPSCs, combination of growth factors, complemented with LacZ, FOXA2 and HNF1α transduction -Matrigel coated plates</td>
<td>80% Albumin positive cells</td>
<td>-AFP positive -Urea synthesis ~50% of PHH -Albumin synthesis ~60% of PHH -CYP inducibility 1.5-50% of PHH -Basal activity panel of 8 CYPs: 1.7%-24% of PHH -LDL uptake -ICG uptake and excretion</td>
<td>Cryopreserved PHHs, single donor, tested 48 hr after plating on collagen coated plates</td>
</tr>
</tbody>
</table>
### Bioartificial livers in vitro and in vivo

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Efficiency</th>
<th>Hepatic functionality</th>
<th>Control PHHs</th>
</tr>
</thead>
</table>
| Takayama 2013 [52] | IPSCs, combination growth factors, complemented with Lin2, FOXA2 and HNF1a transduction           | Not reported | - AFP positive  
- Albumin synthesis ~70% of PHH  
- Urea synthesis ~50% of PHH  
- Baseline CYP3A4 activity comparable to PHH, inducibility <25%  
- Acetaminophen toxicity <40% compared to >90% in PHH                                               | Cryopreserved PHHs, 3 batches, 48 hr after plating                                              |
| Takebe 2013 [84] | IPSCs, 3-step growth factor-mediated differentiation, followed by co-culture with mesenchymal stem cells and HUVECS, upon which liver buds formed  
- Matrigel coated plates                                                                       | 71.9% Albumin positive cells                                                                   | - Albumin synthesis ~100% of PHH  
- >50% of Albumin positive cells are AFP negative!  
- no further testing of in vitro-construct.                                                       | Cryopreserved PHHs, 3 donors, 24 hours after plating                                         |
| Zhu 2014 [91]   | iMPCs (induced endoderm), followed by a 2-step cytokine/growth factor driven differentiation  
- Matrigel coated plates                                                                       | ~65% Albumin positive cells                                                                    | - Albumin synthesis ~50% of PHH  
- Urea synthesis detected, not quantified  
- glycogen positive, PAS staining  
- LDL uptake  
- CYP3A4 and 2C19 activity <5% of PHH                                                             | Cryopreserved PHHs, 24 hrs after seeding                                                        |
| Huang 2014 [92] | iHEPs, HNF4a, FOXA3 and HNF1a transduction and growth factor containing medium  
- Collagen sandwich culture.                                                                    | 36% Albumin positive cells 25.3ALB+ and AAT+                                                  | - AFP undetectable by IHC  
- Albumin and AAT syntheses comparable to PHH.  
- Biliary markers absent  
- LDL-uptake  
- Glycogen positive  
- Inducible expression of several CYPs at the level of PHH  
- Activity of tested CYPs at ~1-6% of PHHs  
- Biliary transporter activity in range of PHHs for several compounds  
- ICG uptake and excretion  
- Transcription of major drug transporters and phase 2 detoxification enzymes >20 to >100% of PHHs  
- IC50 uptake and excretion  
- Glycogen positive  
- LDL uptake                                                                                   | Cryopreserved PHHs from 3 donors, culture period not specified for all assays, directly used after thawing for functional detoxification tests. Function reported to be consistent with previous reports by others |
| Du 2014 [93]    | iHeps, 6 transcription factors  
- Matrigel coated plates                                                                         | 91.7% Albumin positive cells 99.8% AAT positive cells                                         | - AFP negative IHC  
- Albumin synthesis ~70% of PHH  
- Several major CYPs: Expression and activity >25 to >100% of PHH  
- Transcription of major drug transporters and phase 2 detoxification enzymes ~20 to >100% of PHH  
- ICG uptake and excretion  
- Glycogen positive  
- LDL uptake                                                                                   | Freshly isolated PHH control from 2 discarded donor livers, cultured for a non-specified duration. (reason to discard organs not mentioned.) |

*IPS cells were grown on a layer of embryonic fibroblast derived cells of which proliferation was halted with Mitomycin-C-treatment.
AAT: alpha 1-antitrypsin; APF: alpha fetoprotein; CYP: cytochrome p450; ICG: indocyanine green; iPSC: induced pluripotent stem cell; LDL: low-density lipoprotein; PAS: Periodic acid–Schiff; PHH: Primary human hepatocyte
Very recently, two papers were published on directly induced hepatocytes, generated from human fibroblasts through transfections with a combination of LETFs (Table 3). Huang et al. produced highly differentiated HLCs by transducing adipocytes and fibroblasts with a set of 3 LETFs (HNF4a, FOXA3 and HNF1a) and using a defined growth factor-containing medium [92]. These HLCs, named induced hepatocytes (iHEPs), exhibited protein synthesis, biliary excretion indices and inducible expression of a substantial proportion of CYPs at the level of -cryopreserved- control PHHs, and did not express foetal liver markers, indicating they may represent a more differentiated hepatocyte phenotype compared to previously described HLCs. However, not all the parameters that we identified as important in Table1 and section 2 have been tested, most importantly nitrogen- and carbohydrate metabolism were not. CYP activity ranged between ~1% and ~6% of freshly thawed PHHs. iHEPs were restricted in their proliferation and formed a heterogeneous population with approximately 20% well-differentiated cells. Through transduction with simian virus 40 large T-antigen (Tag) the cells could regain their proliferation potential for at least up to 10 passages, however AFP expression was induced and hepatic functionality was substantially lost. Tag is a recognized oncogene and is therefore not suitable for clinical application.

The second paper, by Du et al. reported a similar approach, using a combination of six transcription factors to induce the hepatocyte phenotype (HNF1A, HNF4A and HNF6, ATF5, PROX1 and C/EBPa) and conditional MYC and P53 siRNA expression to accommodate proliferation [93]. This resulted in the efficient generation of HLCs, but these cells were not characterized extensively (Table 3).

Embryonic stem cells
Embryonic stem cells can be similarly differentiated into HLCs as iPSCs [52, 82, 85, 87, 90, 95]. There are, however, two disadvantages to HLCs derived from embryonic stem cells compared to iPSCs. Firstly, for applications that require cells from a range of genetic backgrounds with established phenotypes, embryonic stem cells are unpractical because their donors’ mature phenotype is unknown. Secondly, they are the subject of ethical controversy, as they originate from human embryos. Because of these issues we believe that embryonic stem cells are not the biocomponent of choice for clinical and in vitro BAL application.

In conclusion, human adult stem cell derived HLCs do not offer benefits over other stem cell sources in terms of availability or differentiation potential, and are therefore not the most promising candidate biocomponent. Embryonic stem cells share these disadvantages and additional ethical concerns make application of this biocomponent unrealistic. iPSC derived HLC technology has not yet developed into the stage that it is suitable for any BAL applications, but the ability to host hepatitis C virus and maintain donor phenotype characteristics are promising. iHEPS generated through lineage reprogramming are a
promising source of hepatocytes for *in vitro* BAL systems preferably based on patient-specific cell sources, but several difficulties need to be overcome before they can be successfully applied. The main problems are the lack of proliferative capacity and the heterogeneity of the cultures. In addition, although the cells exhibit substantial drug metabolism capacities, they are still not on par with PHHs, and nitrogen and carbohydrate metabolism data are still lacking. The use of BAL technology could aid in further differentiation of these cells [6]. Due to their limited proliferative capacity and complex composition of culture media these cells are more suitable for *in vitro* BAL application than for clinical BALs.

**Co-culture**

Next to hepatocytes, which represent the major proportion of human liver cells, there are other cell types that may be valuable to use in a BAL to support hepatocyte differentiation and add the dimension of cell-cell interaction to *in vitro* applications. Hepatocyte functionality and phenotypic stability can be improved in BAL systems by direct co-culture with mesenchymal stem cells, hepatic stellate cells and endothelial cells [96, 97], as well as through paracrine action of stellate cells [7]. These are, however, all primary cells, posing similar challenges in terms of availability as PHHs, making them highly unpractical for BAL application, with the exception of mesenchymal stem cells, which still have *in vitro* proliferative capacity [98].

Takebe *et al*. have reported that co-cultured iPSC derived mesoderm cells, human vein umbilical cord cells (HUVEC) and mesenchymal stem cells spontaneously differentiate into vascularized liver buds, with the most differentiated phenotype of IPS-HLCs reported as of yet [84]. Kupffer cells, the resident liver macrophages, as well as circulating immune cells have been proposed to play an important role in some forms of DILI [25], a hypothesis strengthened by the observation that pro-inflammatory cytokines and drugs known to cause idiosyncratic liver toxicity, synergistically induced hepatocyte damage *in vitro* [99]. However, at present, to our knowledge there are no suitable *in vitro* models available for these and other immune reactions in the liver.

**CONCLUSION**

BAL devices hold the promise to meet demands currently left unmet by alternative systems for both clinical and *in vitro* application. The choice of a BAL biocomponent depends on the intended application. The cell line HepaRG is currently the most promising biocomponent for clinical BAL application. For *in vitro* applications, there is no cell type that meets all demands, since PHHs are instable and have a low availability, HepaRG cells reach a high differentiation grade, but come from a uniform genetic background, and stem cell derived
HLCs show promising results, but their limited differentiation grade and proliferation capacity need to be overcome.

**EXPERT OPINION**

Until now, a major problem with the generation of fully matured hepatocytes from expandable cell sources suitable for any BAL application is the incompatibility between cellular processes relating to proliferation and hepatic differentiation. The development of HLCs has deepened our insight into these processes. iHEPs generated by Huang *et al.* did not express biliary or foetal markers, but induction of proliferation by transduction with an oncogene impaired differentiation grade [92]. Du *et al.* circumvented this issue of apparent mutual exclusiveness of proliferation and terminal differentiation by conditional RNAi expression [93]. In human developmental physiology, hepatocytes go through many intermediate stages from blastocyst to hepatocyte. Each stage is characterized by up- and downregulation of different transcription factors. We believe that in a similar way controlled phased expansion and differentiation can be the next step to produce highly differentiated hepatocytes now that the molecular mechanisms driving hepatic lineage development are progressively being unravelled. Advanced conditional expression systems are becoming increasingly available and can be exploited to temporarily overexpress transcription factors or inhibit their expression by siRNA. In addition, the role of growth and differentiation promoting factors becomes progressively clear. As their production increases, the costs will decrease, allowing their temporary addition to culture media to drive or inhibit specific phases of the cell production process.

No gold standard for the hepatic differentiation status exists, and the degree of characterization required depends on the aim of the study. Many reports on newly developed HLCs and hepatocyte cell lines only include a limited characterization, making it difficult to fully appreciate the differentiation grade and applicability in BALs. We have therefore summarized which parameters are, in our opinion, necessary to evaluate a biocomponent for BAL applicability in Table 4.

Two points that are especially relevant, although often neglected, are ammonia detoxification and the use of proper reference PHHs. Urea production is often used as a parameter for ammonia detoxification. Sole urea production is, however, of limited value because urea can be a product of arginase activity without involving the complete urea cycle that detoxifies ammonia in an energy dependent way [27]. Heavy isotope ammonia tracing is required to assess urea cycle activity. As described in section 5, the use of high quality reference PHHs is paramount. However, dedifferentiated PHHs have often been used as a reference material or even no PHHs at all, as illustrated by the last column of Table 3.
Table 4. Proposed hepatocyte benchmark for clinical and in vitro BAL-application.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Transcript level</th>
<th>Functional level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immature hepatic markers</td>
<td>Low levels of Alpha fetoprotein, Glutathion synthetase pi, Cytokeratin 19</td>
<td>At least one activity assay to confirm transcript level data</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Panel of proteins, such as Albumin, apolipoproteins and coagulation factors</td>
<td>At least one activity assay to confirm transcript level data</td>
</tr>
<tr>
<td>Detoxification</td>
<td>-Phase 1 and phase 2 enzymes -Apical and basolateral transporters</td>
<td>-At least one phase 1 and phase 2 activity assay to confirm transcript level data</td>
</tr>
<tr>
<td>Nitrogen metabolism</td>
<td>-Urea cycle enzymes -Glutamine synthetase -Arginase 2; high levels are indicative for urea production through arginine degradation, rather than through urea cycle activity</td>
<td>-Ammonia elimination rate to be tested at pathophysiological concentrations. The starting concentration should be in high micromolar to low millimolar range and not higher. -Urea production preferably tested by measurement of mass enriched ammonia conversion into urea (for clinical BAL application)</td>
</tr>
<tr>
<td>Carbohydrate metabolism</td>
<td>Glucose and lactate consumption/production and oxidative phosphorylation</td>
<td></td>
</tr>
<tr>
<td>Differentiation factors</td>
<td>LETFs and nuclear receptors, such as Hnf4a, Hnf1a, Pxr, Car</td>
<td></td>
</tr>
</tbody>
</table>

CAR: Constitutive adrostane receptor; HNF: Hepatocyte nuclear factor; LETF: Liver enriched transcription factor; PHH: Primary human hepatocyte; PXR: Pregnane X receptor.

In this review we defined the relevant hepatic parameters for the various BAL applications, and gave an overview of performances of the available biocomponents. There lies a risk in focusing merely on hepatocyte specific functionality, since other cell types or surrogates may be of added value for both clinical and in vitro applications. Immunomodulation for example, mentioned in section 2 as a potential important therapeutic strategy in the treatment of SLF, is not likely to be achieved using only hepatocytes. An interesting series of experiments in a galactosamine-induced model of ALF in rats, provided evidence that BAL treatment using mesenchymal stem cells, either alone or in combination with rat hepatocytes drastically improved survival as compared to treatment with hepatocytes only or with hepatocyte-
fibroblast co-cultures [96, 100]. In addition, interleukin-2 receptor antagonist (IL-1RA) treatment in galactosamine-treated mice lowered the pro-inflammatory cytokine load and accelerated early phase regeneration after partial hepatectomy [101, 102]. These results should be validated in an additional model of SLF, since it is known that galactosamine-induced liver injury is, at least in part, dependent on cytokine-induced apoptosis. If pro-inflammatory cytokine load indeed is an important contributor to SLF disease severity, BAL therapy should be supplemented accordingly, either through the use of an additional biocomponent such as mesenchymal stem cells, or through the addition of an artificial cytokine scavenger [103].

Taken together, our findings show that BAL systems are evolving to meet requirements posed by different applications. Innovations in stem cell technology will provide a profound understanding of liver development and insight in mechanisms behind liver damage that can be utilized to further optimize the biocomponents for BALs, both for clinical and in vitro applications.
REFERENCES


Bioartificial livers in vitro and in vivo


