
by

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Liver failure, which is the result of liver injury and pathological inflammation, is currently only successfully treated by organ transplantation. However donor organ shortages preclude transplantation for many patients in need. Thus, bioartificial liver systems (BALS) are being developed as a bridge to transplantation, or to create an environment conducive to liver regeneration. Hepatocytes, the main functional cells of the liver, are the cells of choice for BALSs, but in standard conditions ex vivo, they rapidly suffer from a reduction of their functionality and viability. Coculture with stromal cells, for example bone marrow mesenchymal stromal cells (BM-MSC), has been shown to improve, and extend, hepatocyte function ex vivo up to 21 days. But, only small numbers of BM-MSCs can be harvested from adult volunteers. We have previously described an alternative, more plentiful, source of MSCs — human umbilical cord perivascular cells (HUCPVC) — that are easily expanded and non-alloreactive. Our hypothesis was that HUCPVCs are putative stromal cells for hepatocytes. Our results show that HUCPVCs improved hepatocyte albumin secretion, urea synthesis and maintained hepatocyte cytochrome activity and the expression of hepato-specific genes.
Furthermore, there was a net proliferation of hepatocytes, which were polarized in coculture with HUCPVCs, as judged by functional bile canaliculi that were present for up to 40 days. We found that both soluble and non-soluble factors contributed to these effects, while neither was able to allow net proliferation individually. Moreover, HUCPVCs expressed both hepato-trophic and anti-inflammatory factors, at different levels to BM-MSCs, indicating the potential for differential hepato-therapeutics. We conclude that HUCPVCs are putative stromal cells for hepatocytes; they improve hepatocyte functionality, polarity, morphology and net proliferation, and thus present an opportunity for the improvement of both BALS function and liver therapy.
Acknowledgments

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As a requiem, I would also like to thank the rats that gave their life to the different experiments performed, some of which could not even be included within this written report.

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<th>Description</th>
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<tbody>
<tr>
<td>AFP</td>
<td>α-fetoprotein</td>
</tr>
<tr>
<td>ALF</td>
<td>Acute liver failure</td>
</tr>
<tr>
<td>AOCLF</td>
<td>Acute-on-chronic liver failure</td>
</tr>
<tr>
<td>BALS</td>
<td>Bioartificial Liver System</td>
</tr>
<tr>
<td>BC</td>
<td>Bile Canaliculi</td>
</tr>
<tr>
<td>BM-MSC</td>
<td>Bone Marrow Stromal Cell</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>CCAAT-enhancer-binding protein alpha</td>
</tr>
<tr>
<td>CFU-F</td>
<td>Colony Forming Unit-Fibroblast</td>
</tr>
<tr>
<td>COL-I</td>
<td>Collagen type I</td>
</tr>
<tr>
<td>CYP3A</td>
<td>Cytochrome P450 isoform 3A</td>
</tr>
<tr>
<td>DCN</td>
<td>Decorin</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth Factor</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal Bovine Serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
</tr>
<tr>
<td>FN1</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>hBM-MSC</td>
<td>Human BM-MSC</td>
</tr>
<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>HNF</td>
<td>Hepatocyte Nuclear Factor</td>
</tr>
<tr>
<td>HSC</td>
<td>Hematopoietic Stem Cell</td>
</tr>
<tr>
<td>HUCPVC</td>
<td>Human Umbilical Cord Perivascular Cell</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-Gamma</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>ITS</td>
<td>Insulin-Transferrin-Selenium</td>
</tr>
<tr>
<td>JAG1</td>
<td>Jagged1</td>
</tr>
<tr>
<td>KC</td>
<td>Kupffer cell</td>
</tr>
<tr>
<td>KITLG</td>
<td>Stem Cell Factor</td>
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<tr>
<td>MSC</td>
<td>Mesenchymal Stromal Cell</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>NP</td>
<td>Non-parenchymal</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative RT-PCR</td>
</tr>
<tr>
<td>rBM-MSC</td>
<td>Rat BM-MSC</td>
</tr>
<tr>
<td>RLEC</td>
<td>Rat liver epithelial cell</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SC</td>
<td>Stellate Cell</td>
</tr>
<tr>
<td>SEC</td>
<td>Sinusoidal Endothelial Cell</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>TAT</td>
<td>Tyrosine Aminotransferase</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission Electron Microscopy</td>
</tr>
<tr>
<td>TGFA</td>
<td>Transforming growth factor alpha</td>
</tr>
<tr>
<td>TGFB</td>
<td>Transforming growth factor Beta</td>
</tr>
<tr>
<td>TNFa</td>
<td>Tumour Necrosis Factor alpha</td>
</tr>
<tr>
<td>TO</td>
<td>Tryptophan 2,3-dioxygenase</td>
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1. CHAPTER1: Introduction
This thesis focuses on exploring a putative source of supportive cells for ex-vivo culture of hepatocytes, within the context of the requirements of a bioartificial liver system (BALS). Thus, in this Introduction, after explaining why ex-vivo hepatocyte culture is necessary, and for which diseases such an approach is relevant, a brief summary of the current BALS state-of-the-art is provided together with an explanation of how current BALS technology can be improved. To provide a context for these ex-vivo strategies, the gross and cellular structure of the normal liver is reviewed detailing the function of each cell type that contributes to the organization of the hepatocyte environment. After this, the general properties of mesenchymal stromal cells (MSC) are described, since human umbilical cord perivascular cells (HUCPVC), the cells being explored herein, are MSCs. In particular the hepatotrophic properties of MSCs are described. Finally the current methods of hepatocyte cell culture are reviewed, in order to provide a perspective for the rationale of each of the individual experiments described in the later sections of this thesis.

1.1. Liver Failure

Upon liver injury, by any means, including mechanical, pathogenic or chemical, a localized inflammatory response is developed. This response eliminates the injuring agent and the necrotic tissue so that regeneration can happen. In some cases the inflammatory response can get out of control, being so much, that it actually causes injury to the liver. Depending on the intensity of the inflammation it can lead, or contribute, to extensive damage of the liver, causing acute liver failure (ALF) or it can be moderate but unresolved and therefore cause chronic injury which ultimately leads to cirrhosis (1).

There are two stages of liver insufficiency, a compensated stage and a decompensated stage. The compensated stage is asymptomatic, except for the possible presence of jaundice, and many times the signs of its presence can only be detected by liver function tests (galactose, indocyanine green, monoethylglycinexylidide (MEGX)) as routine laboratory tests may not show any significant abnormalities (2). In the decompensated
stage many symptoms can be obvious, including jaundice, coagulopathy and encephalopathy (3-5). The decompensated stage can take years to just days to establish and, depending on the time required for this onset, it is classified in two main types: ALF and acute-on-chronic liver failure (AOCLF). In Canada between 2003-2007, approximately 1.1% (6) of deaths occurred due to AOCLF, which usually tends to be present in the form of decompensated cirrhosis (2) which is the reason why, cirrhosis and its mechanisms are subject of intensive research. This is due to the possibility of reversal and prevention through various techniques, one of them being cell therapy. Below we address ALF and cirrhosis in further detail.

### 1.1.1. Acute Liver Failure

ALF is an illness in which liver function is suddenly lost without an underlying hepatic pathology (2, 7) causing coagulopathy and encephalopathy within a short period of time (5). There are three sub-classifications of ALF: hyperacute, acute and subacute. In hyperacute, encephalopathy develops within 7 days of the insult (survival rate 30-40%), in acute within 8-28 days (survival rate 5-10%) and subacute in 29-72 days (survival rate 10-20%) (2, 5). To date the known causes of ALF are viral hepatitis, drugs and toxins, metabolic anomalies and ischemia but in many cases the cause is unknown (Figure 1) (5, 7). At the cellular level, in order for ALF to develop there has to be a critical degree of cell death that cannot be compensated by regeneration (8). Two mechanisms of cell death can occur in ALF, apoptosis and necrosis. Apoptosis is characterized by the lack of secondary inflammation, which is caused by necrosis due to the release of cellular contents. Necrosis primarily happens in drug induced ALF and apoptosis has been seen to be preponderant in cases of ischemia and fulminant Wilson’s disease (8).

ALF is associated with a systemic inflammatory response syndrome (SIRS), regardless of whether there is any infection (9). The serum of ALF patients usually contains high levels of pro-inflammatory cytokines: Interleukin-1, tumour necrosis factor α (TNFα) and Interleukin-6 (IL6) (8, 9). Both TNFα and IL6 are also very important in triggering hepatic regeneration, but in ALF, TNFα can act as an apoptotic inducer in hepatocytes (8). Also, it has been documented that inflammatory cytokines like interferon-γ, IL12 and
IL5 exacerbate ALF and anti-inflammatory cytokines like IL4, IL10, IL11, IL13 can protect the liver from injuries that can lead to ALF (8).

The current treatment of choice for ALF is emergency liver transplantation (7) that can significantly increase patient survival rate (5). Today, the main cause of death after ALF is cerebral oedema and sepsis, but renal failure, bacterial and fungal infections, and respiratory impairment with the requirement of mechanical ventilation are also common (7).

![Figure 1: Causes of ALF in the United States between 1998-2007. This distribution is different in other regions; in Asia and Africa the main cause is viral hepatitis including HEV, HAV and HBV. Taken from Lee et al. 2008 (7) with permission from John Wiley and Sons.](image)

**1.1.2. Cirrhosis**

As previously mentioned, liver injury results in liver inflammation and regeneration however repeated healing attempts, due to chronic injury, may eventually lead to degeneration into scarring which causes liver fibrosis (10). In liver fibrosis the injured liver tissue is either surrounded or replaced by fibrous tissue (10, 11). If fibrosis advances further, it becomes cirrhosis where the vascular components are also compromised (11). In cirrhosis, the space of Disse (Figure 6) is filled with collagen type IV and laminin, creating the usual endothelial basement membrane; also there is an increase of collagen type I, II, V, VI and fibronectin (FN1), which makes the space of Disse abnormally wider and denser (12). Liver stellate cells (SCs – see Section 18 for more details) are considered
the main mediators of liver fibrosis and therefore cirrhosis. In fibrosis and cirrhosis, SCs are activated, due to the release of cytokines from the recruited inflammatory cells, the damaged and regenerating hepatocytes and other cells (10); this turns them into proliferative, contractile, chemotactic and fibrogenic (13), secreting high amounts of collagen type I, III, IV, laminin, and loosing the majority of their lipid vacuoles (12). Many of the liver sinusoidal endothelial cells (SECs) loose their fenestrations and form tight junctions between them, creating normal capillaries, thus impairing the communication of hepatocytes with the blood flow (11, 12). Also, histologically, hepatocytes are found in nodules (Appendix 1) surrounded by fibrous tissue composed of collagen type I, III and VI and FN1; in many cases, hepatocytes are more than one cell away from the vessels, creating further complications for them to perform their functions (11, 12).

Cirrhosis is usually in the compensatory stage until the late stages of the disease where decompensation occurs (11). However in both decompensated and compensated cirrhosis there is impaired liver function, portal hypertension, and the possible development of hepatocellular carcinoma (HCC) (11). In decompensated cirrhosis, patients can suffer from jaundice, coagulopathy, bleeding, ascites and renal dysfunction and encephalopathy (2-4).

The main causes of cirrhosis vary, in the West the two main causes are alcohol and hepatitis C; in the East hepatitis B (11). Cirrhosis can also be caused by immune mediated damage, genetic abnormalities, biliary disease and non-alcoholic steato-hepatitis (10). At present cirrhosis is being treated by first removing the detrimental stimulus (if possible) and using anti-virals (in the case of viral hepatitis) (10). However, it is still unknown whether cirrhosis is completely reversible — as fibrosis is — thus the only proven cure today is liver transplantation, which is available to a selected patient group (10, 11). The main causes of death of cirrhotic patients who do not receive liver transplants are liver failure, HCC, or bleeding (3).
1.2. Bioartificial Liver

End-stage liver disease, as previously mentioned, is usually treated by liver transplantation, however donor organ shortages preclude transplantation for many patients in need; only 30% of the patients in the waiting list receive a transplant and many others are not even eligible to be on the waiting list (14). Many patients with acute liver failure die while waiting for a transplant and those with chronic disease often deteriorate so much that they suffer a high mortality rate after transplantation. Due to the regenerative potential of the liver, a temporary support with hepatic supplementation systems, i.e. bioartificial liver systems (BALS), would make many transplantations unnecessary or decrease deterioration while bridging for transplantation (14-17). For 30 years, BALSs have been in development, using both cells and synthetic components (18). Because of the main role of hepatocytes in the liver, their ex-vivo culture is of extreme importance for the development of BALSSs (19, 20). Furthermore, when an analysis is done on the known cells that can provide liver supplementation, primary hepatocytes predominate. The current human liver cells lines, which include the C3A line and immortalized adult and foetal hepatocytes, have many drawbacks. The C3A cell line, derived from a HepG2 clone, despite being currently used in a BALS clinical trial (ELAD), fails when compared to porcine hepatocytes, having a significantly lower metabolic activity, specifically with respect to ammonia elimination which is crucial for BALSSs (21). Immortalized adult hepatocytes, when reversed to normality, have been shown to have poor RNA expression of hepatic genes; the levels of albumin, α-1-antitrypsin and transferrin are only 0.1% of the levels of adult human hepatocytes (17). While foetal hepatocytes can proliferate through 30-35 doublings, immortalized foetal hepatocytes can proliferate theoretically indefinitely while maintaining a similar functionality (17). However, the metabolic and synthetic capacity of foetal hepatocytes is significantly lower, being significantly impaired in ammonia elimination, compared to adult hepatocytes (22-24). Thus, since stem cell technology still has much work to do to create mature hepatocytes, the most promising cells to date are primary hepatocytes. As discussed previously, primary hepatocytes do not maintain their functions adequately ex vivo, but for the time that they do, they provide adequate metabolic activity for BALSs,
thus improvement of both their maintenance and expansion ex vivo are important aims. To date, the use of human hepatocytes has been limited in BALSs, due to the fact that at least $1-2 \times 10^{10}$ (15, 17, 21) hepatocytes are required and it is a challenge to have them available when needed (17). Another drawback is that the human hepatocytes used for BALSs have never been acquired from uncompromised livers (21). Without doubt human hepatocytes are ideal for BALSs as they present very low biosafety issues and also have the best level of hepatic biofunctions for human requirements; Poyck et al. (23) found that the ammonia elimination and urea synthesis of human hepatocytes was 2-3 times higher than that of porcine hepatocytes. Despite the advantages of using human hepatocytes, due to their limited availability, porcine hepatocytes have been the hepatocytes of choice for most of the current BALSs due not only to their availability but also their low cost, similar physiology to humans and well-established isolation methods (16, 21). It is important to mention that porcine hepatocytes, being xenogeneic, generate an immune response from the host, as noticed by the significant increase of pig antibodies and factors of the complement system in serum of patients treated more than once. Due to this, porcine hepatocytes are attacked, primarily through the complement system (17, 21). The other disadvantage and a major concern is pig endogenous retrovirus (PERV), although there have been no case reports of PERV infections caused by the direct exposure of human plasma to porcine cells (17, 21).

Because of the above mentioned concerns, BALSs need to be designed so that cells are isolated from the host immune system while maintaining an adequate mass transfer between the BALS cells and the patients plasma (18, 25). Currently BALSs attempt to improve the maintenance of hepatocytes so that they perform most of their functions. However, when it comes to select the most important function, ammonia elimination and urea synthesis is always chosen, as ammonia is highly toxic to the central nervous system (15, 18).

The 4 main types of bioreactors used in BALSs to date are hollow fibre cartridges, flat plate, packed beds and encapsulation systems (21, 25)(Table 2). The current BALSs have so far shown to significantly improve many physiological parameters and clinical symptoms but there has not been a significant improvement in the survival times (21).
The BALSs that have so far being tested in clinical trials are the Academic Medical Center-Bioartificial Liver (AMC-BAL), the Bioartificial Liver Support System (BLSS), the Extracorporeal Liver Assist Device (ELAD) and the Modular Extracorporeal Liver Support (MELS). In each of the systems blood is treated differently before reaching hepatocytes as shown in Table 1, thus potentially exposing hepatocytes to different stimuli. Also, each one has different cell components and quantities (Table 2).

Table 1: Blood Pre-Processing in BALSs

<table>
<thead>
<tr>
<th></th>
<th>Plasmapheresis</th>
<th>Hemofiltration</th>
<th>Dialysis</th>
<th>Adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepatAssist</td>
<td>X</td>
<td>X</td>
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<td>X</td>
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<tr>
<td>ELAD</td>
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<td>BLSS</td>
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<td>MELS</td>
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<tr>
<td>AMC-BAL</td>
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Plasmapheresis, separation of plasma; Hemofiltration, filtration based on a medium permeability membrane (15 kDa<MWCO<70 kDa); Dialysis, diffusion through a low-to-medium permeability membrane. A buffered dialysate present in the extracapillary space is used for the removal of water-soluble toxins; Adsorption on various substrates to remove protein-bound toxins, such as bilirubin or bile acids. MWCO, molecular weight cut-off. Legend and table after Carpentier et al. 2009 (26).

As seen in Table 2, BALS treatment times are limited to less than a week even when cell lines are used. In the most complex type of bioreactor, the MELS, hepatocytes are attached to a surface coated with Matrigel and they are in coculture with liver non-parenchymal (NP) cells; in this bioreactor hepatocytes can last more than 21 days in good condition as judged by electron microscopy (27), but albumin synthesis at day 21 has been reduced by 7 times in relation with the first day (28) and urea synthesis is significantly reduced from day 11 (29). Thus, due to the fact that BALSs have to be tested before being used, for instance up to 21 days of stand-by phase in MELS (30), and liver treatment times ought to be prolonged, it is important to continue the search for methods that improve the approximation of the hepatocyte culture environment to the niche in which they reside in vivo. One of the ways in which this can be achieved is the
use of improved coculture systems, due to the fact that to date liver stromal cell types have not been able to fully serve as stroma ex vivo, mainly due to their phenotypic change when extracted from the liver, e.g. SECs loose their fenestrations and SCs become activated.

<table>
<thead>
<tr>
<th>Table 2: Biological components in BALSs (Carpentier et al, 2009 (26) and Wang et al, 2010 (21))</th>
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<tbody>
<tr>
<td><strong>System</strong></td>
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<tr>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>HepatAssist</td>
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<td>ELAD</td>
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<td>AMC-BAL</td>
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</tbody>
</table>

Additionally, immunogenicity of BALSs is one issue that has to be addressed as it is one factor that can significantly affect their effectiveness as has already been seen with xenogeneic systems (discussed previously in this section). With allogeneic BALSs an immune response can also be induced despite the low immunogenicity of hepatocytes, which lack the expression of MHCII and have low expression of MHCI (31-33). Hepatocytes have been seen to cause immune responses allogeneically both in vitro and in vivo (33, 34); also their immunogenicity can change in hepatitis (32). Due to the fact that MSCs are anti-inflammatory (see section 1.4 for a better description of MSC properties) and can support hepatocytes (see section 1.4.2), they can be ideal for not only the maintenance of an adequate environment for hepatocytes but also for preserving a non-inflammatory environment. This can be beneficial for the BALS hepatocytes and also can help in the recovery of the host liver by the released factors.
1.3. Liver

The liver is the second largest organ (after the skin) and the largest gland in the body, being both endocrine and exocrine (35, 36); it comprises ~5% of the mammals body mass (37). In human adults it account for 2% of the body weight while in children it is 5% (35, 36). It is supplied with oxygenated blood by the hepatic artery. Blood from the gut comes through the portal vein which is later mixed with arterial blood (38) in order to be processed while being both nutritional and oxygenating to the cells along the sinusoids. All this blood then ends up exiting from the liver through the 3 hepatic veins, the right, middle and left hepatic veins; which disembogue into the inferior vena cava, except for Segment I that drains directly into the inferior vena cava (Figure 2) (35, 39). The liver is divided topographically into 4 lobes and functionally into 8 segments (35, 39). In 1954, Couinaud described the liver based on 8 independent segments, each receiving a portal venous branch, hepatic arterial branch and a bile duct radicle, each also having its own venous drainage (Figure 2) (39). This description has been very important for surgery as it allows for the removal of each segment without affecting the others. The equivalency of each segment in relation with the topographical anatomy of the liver is: segments II, III make the left lobe; segments V, VI, VII and VIII make the right lobe; segment IV is equivalent to the quadrate lobe and segment I to the caudate (39).

To date there is no agreement upon a functional unit of the liver as none of the approximations have been able to account for all the functions that it accomplishes (35). One useful concept is the one of the liver lobule described by Kiernan in 1833. Its shape is usually hexagonal, when cross-sectioned, and measures 0.7x2mm (Figure 3). Around 24 hepatocytes separate the portal tract branch from the central vein. The portal tract is covered by concentric layers of hepatocytes that are only interrupted by the links between portal vessels and the sinusoids. Hepatocytes are organized in plates which are 1-2 cell thick in human adults and are separated one from the other by the capillary sinusoids which converge in the central vein (35).
Figure 2: Segmental anatomy of the liver as described by Couinaud. The number of segments is organized in a clockwise manner. Figure from Radgray TM (40)

Figure 3: Liver lobules. Schematic diagram of liver lobules (left) (figure from Gray's anatomy (41) used according to Elsevier Policies). Liver lobule structure (right) (modified drawing from http://www.daviddarling.info/encyclopedia/L/liver.html (42), used with authorization from David Darling).

The parenchymal cells of the liver are considered to be the hepatocytes. Hepatocytes, which are further described in section 1.3.2.1, perform most of the liver functions and account for 80% of its volume and 60-70% of the cell number (35). The NP cells include SCs, SECs, Kupffer cells (KCs), cholangiocytes, Pit cells and oval cells. Most of the cells are closely associated with the sinusoids except for cholangiocytes, which are located in
bile ductules and ducts, and the canals of Hering (Figure 3, right); and oval cells, which are mainly found in the canals of Hering (35, 43). The sinusoids are coated by SECs which are fenestrated, these fenestrations in addition to the lack of basement membrane allows for an easy but selective exchange of substances between the sinusoidal lumen and the space of Disse and hepatocytes. The space of Disse is a small space between hepatocytes and SECs, having a width of 0.2-0.5 um (35) and containing extracellular matrix (ECM) proteins such as collagens I, III, V, VI, FN1 and syndecan (44). SCs reside in the space of Disse; and KCs and Pit cells are normally found within the sinusoids (Figure 6).

The liver is a very important organ to maintain homeostasis. It is involved in a myriad of metabolic functions through hepatocytes (see section 1.3.2.1). The liver also produces bile. Primary bile is produced by hepatocytes and secreted into the bile canaliculi, which are formed by the canalicular membranes of two adjacent hepatocytes (Figure 6) (45). The bile canaliculi later disemboque the primary bile into the bile ductules, through the canals of Hering, which are canals that are lined by both hepatocytes and cholangiocytes. Bile ductules are completely lined by cholangiocytes but they do not have basement membrane and as the canals of Hering, they are just another link between the bile canaliculi and the interlobular bile ducts, which have a diameter of 15-20 um. These bile ducts then, as with a river, continue increasing diameter as they fuse, until they get to be the large intrahepatic hilar bile ducts (35). During the time when the primary bile goes through the bile ducts, it is transformed into (mature) bile by the lining cholangiocytes as briefly described in section 1.3.2.5.

The liver has an incredible regenerative potential despite the fact that most of its cells are highly differentiated and quiescent (46, 47). Unlike other organs where regeneration is based on resident stem cells or progenitors, the liver usually uses all its cells to regenerate (46, 48) unless hepatocyte proliferation is impaired (35, 43, 48); in regenerating livers of rats it has been seen that more than 95% of hepatocytes do proliferate as indicated by their intake of [3H]thymidine (48). To date, it is accepted that at least 40% of the human liver is necessary for a partial liver transplantation (49). However, in rats it has been seen that it is possible to undertake a ~90% partial hepectomy while having 100% survival
and it is also possible to perform successful transplantations with only 20\% of the liver mass (49). After this, while the absent lobes do not reform, the liver mass does start increasing, undergoing what is technically known as compensatory hyperplasia (46, 47). In the case of rats, the hyperplasia is so fast that after one week of either 80\% partial hepatectomy or transplant of 20\% of the liver mass, the liver augments its liver mass back to \(\sim 100\%\) of the original (49). Liver hyperplasia can occur not only due to cellular resection but also due to other causes where cells are lost or there is functional inadequacy, i.e. infections, toxic or physical injury, diabetes (46). The liver always aims to reach a size where it is adequate to maintain homeostasis, thus depending on its requirements it can grow but also shrink, as has been seen by transplantations of livers of big dogs into small dogs (47), where the liver reduces its size by inducing cell apoptosis (46).

1.3.1. Liver Development

In human development, after one week following conception, the multicellular structure formed starts gastrulation, showing a distinct division giving rise to the early endoderm and the ectoderm (50). Then on the third week (50), 3 germ layers can be distinguished, the ectoderm, endoderm and mesoderm, at which time it is considered that embryogenesis starts and blastogenesis is completed (51). Embryogenesis is when organs and systems are formed, and this period lasts until the ninth week (50) when the foetal stage or foetogenesis starts, lasting until birth (50, 51).

The liver starts developing by a process called liver specification in which mesoderm factors induce the adjacent endoderm to start hepatogenesis (35). This induction has been shown to require at least fibroblast growth factor (FGF) signalling from the cardiogenic mesoderm and bone morphogenetic protein (BMP), especially BMP-4, coming from the septum transversum mesenchyme in order to induce hepatic specification and promote the expansion of the liver bud (36, 52, 53). It has also been seen that TGF\(\beta\) regulates the differentiation while WNT signalling restricts hepatic specification to the anterior endoderm (36). Thus, around the third week of gestation it is already possible to distinguish a thickening of the endoderm of the ventral wall of the foregut, which is
called the liver bud (35, 51) (Figure 4). Some of the cells contained in the liver bud already show signs of being hepatoblasts, expressing hepatic genes: Albumin, AFP, HNF4α, transthyretin and retinol binding protein (35, 36).

As hepatoblasts continue receiving signals from the mesoderm through various cytokines including, FGF, epidermal growth factor (EGF), hepatocyte growth factor (HGF), TGFβ, TNFα and IL6 (53); they continue proliferating which then makes them bulge into the surrounding stroma and give rise to the hepatic diverticulum around day 22 of gestation (35, 51) (Figure 4). The pressure caused by this event, eventually breaks the surrounding basement membrane, which contains laminin, nidogen, collagen IV, FN1, and heparin sulphate proteoglycans (36). This thus allows the invasion of the septum transversum, around days 26 to 30, by cords of hepatoblasts (35, 36, 52) (Figure 4). Interestingly the migrating hepatoblasts, despite still keeping their bipotentiality, express high levels of HNF4α and C/EBPα, which are regulators of hepatocyte differentiation, while HNF6 and HNF1β, which are regulators of cholangiogenesis, are very low (36). Thus the cranial region of the diverticulum later gives rise to the liver with the intrahepatic bile ducts and the caudal region gives rise to the gall bladder, the cystic duct and the common bile duct (35, 51).

Figure 4: Schematic of liver bud formation and later invasion of the septum transversum (STM).
Foregut Endoderm, End (pink); Heart, He (red); Liver bud, Lb (green); Vascular endothelial cells, E, (black); STM (Magenta). Approximate week in human gestation is shown. Modified schematic from Si-Tayeb et al. 2010 (36) used with authorization from Elsevier.
As hepatoblasts continue invading the stroma they further mature and start forming sheets and cords, called plates, which at this stage are 5-6 cells thick but at birth will thin down to a thickness of 2-3 cells (35). These plates form following the arrival of stromal capillaries from the vitelline veins of the yolk sac, which later fuse to form the portal vein (35, 51). As this happens, angiogenesis from these vessels and possibly some vasculogenesis (35, 36), leads to the formation of the sinusoids, being the first blood vessels of the liver (36). Eventually the endothelial cells of these newly formed capillaries acquire the phenotype of mature SECs, an event that is correlated with a change in the surrounding ECM (36). It is noteworthy to mention that signals from endothelial cells are crucial for liver development; a factor secreted by endothelial cells, Wnt9a, has shown to be a hepatoblast mitogen and a regulator of global liver morphology (53).

![Liver development timeline](image)

**Figure 5: Liver development timeline.**
Red lines depict liver development stages. Black lines show the stages for mouse development and blue lines for human. Taken from Kung et al. 2010 (53).

At this stage, the embryo is around the sixth week of gestation, the liver already has two clearly defines lobes (35) and the first hematopoietic stem cells (HSCs) appear giving rise to the start of the hematopoietic function of the liver (51, 54). This function becomes clearly evident in weeks 8-10 (51, 54) until it reaches a peak in months 6 and 7 and then it is repressed while the bone marrow gradually takes over the hematopoietic function (35, 51). The liver completely stops haematopoiesis in the first week after birth however
HSCs may persist in the liver for the lifetime of the individual (35, 55). While the liver serves as a site for haematopoiesis, hematopoietic cells are located outside the vessels in contact with the liver parenchyma while contributing to liver development by cytokine signalling (35, 52); CD45 hematopoietic cells express oncostatin M, which along with HGF (53), contributes to hepatocyte maturation and eventually makes them unsuitable to support haematopoiesis (36, 56, 57).

In the sixth week of gestation, canaliculi begin to appear between immature hepatocytes; the ductal plate, a layer of ductal cells surrounding the periportal mesenchyme, starts to form from hepatoblasts (35, 51) that differentiate under the control of the Jagged-Notch pathway (58). The first SCs may also appear in this week, deriving from mesothelial cells coming from the septum transversum and proepicardium (35, 36, 51). Around the third month, KCs develop, probably originating from the yolk sac or HSCs (35, 51); hepatocellular bile formation starts (35) and the ductal plates, which are already two cells thick, remodel into interlobular bile ducts and excess bile ducts are degraded (35, 51). Later in week 28, the external bile duct formation is completed, while the intrahepatic bile ducts continue maturing for a few weeks after birth (51).

### 1.3.2. Liver Cell Types

![Liver cell types and their relative location in the liver.](image)

Figure 6: Liver cell types and their relative location in the liver.
1.3.2.1. Hepatocytes

Hepatocytes are the main functional cell of the liver requiring for their functionality and regeneration the support from the liver stroma (15, 19, 59), which contains all the NP cells with which hepatocytes interact (Figure 6). The parenchymal cells of the liver perform a myriad of functions having influence over the metabolism of proteins, lipids, carbohydrates and xenobiotics.

Hepatocytes perform aminoacid and protein degradation and synthesis. They also produce urea, a less toxic compound than the ammonia resulting from aminoacid degradation both in hepatocytes and other cells. They are able to produce any of the non-essential aminoacids and degrade most of them (except leucine, isoleucine and valine) (35). They synthesize numerous proteins: half of them being structural cell proteins and enzymes; the other half being secreted proteins including hormones such as insulin-like growth factors, angiotensin and thrombopoietin (36); and most plasma proteins, i.e. albumin, excluding immunoglobulins (35).

The role of hepatocytes in lipid metabolism include the oxidization of triglycerides in order to convert carbohydrates into fatty acids and triglycerides; they also synthesize cholesterol, phospholipids and bile acids, the most important components of bile not including bilirubin, and a large number of lipoproteins (35). It is important to note that the most important exocrine secretion of the liver (36) and thus of hepatocytes is bile; which is not only useful for excretion but, importantly, it also plays roles in facilitating the absorption of fat and fat soluble substances by the gut, neutralization of gastric juice, activation of pancreatic enzymes and stimulation of gut motility (35).

In addition, the liver parenchyma contributes to glucose homeostasis by storing glycogen, being an important reservoir of energy for the body. Thus parenchymal cells are able to both polymerize glucose into glycogen (glycogenesis) and to depolymerize it back into glucose (glycogenolisis) to maintain blood glucose homeostasis (35). They can also synthesize glucose from aminoacids and non-hexose carbohydrates (gluconeogenesis) when necessary (37).
Another very important role of hepatocytes is the metabolism of xenobiotics, i.e. alcohol and drugs, and hormones (35, 37). They can be metabolized in various cell compartments, however the membrane system of the smooth endoplasmic reticulum is where most of the degradation occurs. The main enzymes found there are the cytochrome P450 isoenzymes, which can also be found in the nuclear membrane (35).

Hepatocytes also perform other functions like storage of Vitamin A, along with SCs; vitamin D, E, K, C and the vitamin B group (35, 37). They store several minerals, including copper, iron, potassium, zinc and cobalt (37). Thus, the homeostasis of the whole body is critically maintained by hepatocytes.

1.3.2.2. Stellate Cells

Stellate cells (SC) are also called Ito cells (60), Vitamin A or fat storing cells, lipocytes, interstitial cells and liver specific pericytes (35, 61), being the only liver cells known to be of mesenchymal origin (see section 1.3.1). Their number is close to one fourth of the NP cells of the liver (13, 35) and about 3-10% of the whole liver cells (51, 61). They are located in the space of Disse (Figure 6), thus being in juxtaposition to hepatocytes and SECs. Evidence shows that they play a vital role in both liver development and regeneration (13). Indeed, SCs secrete hepatotrophic growth factors: such as HGF, stem cell factor (KITLG), TNFα, IL6, EGF, epimorphin and pleiotrophin (13, 35); in vitro they have shown to enhance cytochrome activity and albumin secretion (62) and their soluble factors have shown to enhance albumin secretion and urea synthesis compared to monoculture (63). In addition, they produce matrix proteins that have shown to support hepatocytes: FN1, proteoglycans, laminin and collagens (35, 64, 65).

Conversely SCs also play a very important role in the development of liver fibrosis (35). They also perform other functions in the liver: Vitamin A and lipid storage and transport (35, 66); SCs store up to 72% of retinoids (Vitamin A compounds) in the whole body (13). They are involved in the regulation of sinusoidal blood flow, and the assembly and degradation of cellular matrix (35), being the most important collagen synthesizing cells of the liver (64). They also participate in the immunoregulation of the liver, not only
being capable of stimulation inflammatory reactions but also being able to induce immunotolerance (13).

Two very different states have been identified in SCs: an activated and an inactivated state. When a SC gets activated it gets transformed from a retinoid-rich, resting, non-proliferative cell to a proliferative, contractile, mobile and fibrinogeneic cell (13). Activated SCs can become problematic, not only as a cause of liver fibrosis, but later also cirrhosis. A delicate regulation of their activation is therefore needed and it requires cross-communication with both parenchymal and NP cells (13, 35). It is not known however whether SC activation is a response required for liver regeneration (13).

1.3.2.3. Kupffer Cells

Kupffer cells (67) (KC) are the resident liver macrophages accounting for 8-12% of the liver cells (51), however their number can vary significantly not only depending on the anatomical location within the liver but also other systemic factors (35, 68). They eliminate most of the pathogens coming from the portal blood flow; they clear close to 90% of the bacteria (25). It is also believed that they exert paracrine signals on hepatocytes, SCs and SECs (35). Some of the cytokines that KCs can secrete are hepatotrophic factors: IL6, TNFα and HGF (35). Zinchenko et al (68) showed that KCs provide some support to hepatocytes, that does not seem to require contact, but only when the proportion of KC to hepatocytes is low, otherwise KCs can have a negative impact.

1.3.2.4. Sinusoidal Endothelial Cells

Sinusoidal Endothelial cells (SEC) are the cells that coat the liver capillaries, accounting for 15-20% of the total liver cell number (51), but unlike other endothelial cells, they lack a basement membrane (35, 69). They also differ in that they contain open pores, or fenestrae, which make the liver capillaries a selective sieve, protecting the parenchymal
cells and SCs from contact with undesired agents (35) and also allowing the interaction of T cells with hepatocytes (70).

SECs can also uptake blood materials and either degrade them or transfer them to hepatocytes (35). In addition, they catabolize components of the extracellular matrix and in case KCs are not enough, they can become phagocytic (35).

SECs secrete numerous cytokines including TNFα, IL6, HGF and TGFβ (35), all of which can affect hepatocytes (65, 71) and some of which affect SCs (72). Reports have shown that SECs maintain the uptake of LDL by hepatocytes when they are cocultured (73) and also participate in the activation (72) and deactivation of SCs (74).

1.3.2.5. Cholangiocytes

Cholangiocytes, which comprise 3-5% of the cells in the liver, form the bile ductules and the bile ducts and part of the canals of Hering (35), all of which allow the release of bile secreted by hepatocytes into the small intestine (75). Bile duct cells are organized in a polarized monolayer, one face laying on top of basement membrane and the other face facing the lumen of the ducts (76). They control the rate of bile flow and the pH of the bile by secreting water and bicarbonate ion respectively into the lumen (76).

Interestingly, despite their relatively distant localization within the liver compared to SCs, KCs and SECs, cholangiocytes, which can secrete IL6, are able to provide support to hepatocytes in vitro; being able to better maintain albumin secretion, urea synthesis and cytochrome activity compared to monoculture (77).

1.3.2.6. Pit Cells

The name “Pit cells” (78) is given to the hepatic large granular lymphocytes, which functionally act as natural killer cells (NK) (79), being the most cytotoxic of all the naturally occurring NK cells in the body (80). They account for only 10% of the number of KCs (35), accounting for 0.8-1.2% of the whole liver cell number, and are usually
found attached to KCs and the luminal side of the sinusoids, and not present in the space of Disse (35, 79).

1.3.2.7. Oval Cells

Oval cells or hepatic progenitor cells reside mainly in the canals of Hering (35, 43). They are cells that have the capability of rapid proliferation and are able to give rise to hepatocytes and cholangiocytes, thus in cases when hepatocyte proliferation is impaired, they can be activated to aid in liver regeneration (35, 43, 48). However, because of their proliferative capacity and plasticity, which also allow them to differentiate into non-hepatocyte lineages, they have also been implicated in carcinogenesis (43). Their number in the healthy liver is very scarce and their small size (7-10 um) (43) relative to mature hepatocytes (20-40 um) (35), was one of the main characteristics, along with their ovoid shaped nucleus, that led to their identification by Farber in 1956 (43).

1.3.3. Liver Stroma

In the liver, hepatocytes are supported by the hepatic vascular system and the biliary tree, in addition to the connective tissue septa. As previously mentioned, many NP cells have supportive effects on hepatocytes through their secreted cytokines and/or produced ECM. Thus, below, we focus on two important components of the stroma: the ECM and the stromal cells.

1.3.3.1. Extra-cellular Matrix

The ECM accounts for only a small part of the liver, only 0.5% of liver wet weight (81), however it is of considerable importance for the maintenance of the hepatocyte phenotype and as mechanical support. There are three main places where ECM proteins are found: the hepatic capsule, the portal spaces, the space of Disse, and the central space (35, 82). The hepatic capsule is mainly composed of collagen types I, III, V and VI and FN1. The portal spaces contain the interlobular bile ducts, the hepatic artery and portal
vein branches (Figure 3) within an ECM made of collagen types I, III, V, VI, FN1, tenascin and some elastic fibres (44, 83), while each of the vessels and ducts within these spaces are individually surrounded by a basement membrane composed of laminin, collagen type IV, entactin, and heparan sulphate proteoglycans (44).

The space of Disse (Figure 6) is the ECM in direct contact with both hepatocytes and SECs, which unlike other epithelial and endothelial cells lack basement membranes. The space of Disse has FN1, collagen type I, which is organized in fibre bundles that can be mixed with collagen type III, V and VI. Collagen type IV is also found but randomly distributed (13, 44); in the sinusoidal aspect of the space of Disse there is also abundant content of syndecan, a heparan sulphate proteoglycan (44). Some authors also report the presence of laminin, nidogen and perlecan (44, 81, 84), but Matinez-Hernandez and Amenta, 1993 (44) have failed to detect it using different methods. Interestingly, at the early stages of liver regeneration, laminin appears in the space of Disse in a similar fashion to that of the developing liver. This laminin is produced by the proliferating SCs, which peak this production approximately 48 hours after hepatectomy, around the time at which proliferation also peaks (12).

The other place where ECM is primarily found is in the central space where the central vein (Figure 3) is surrounded by a basement membrane made mainly of laminin, collagen type IV and perlecan; this is then surrounded by ECM containing FN1, collagen type I, II, VI and some V (44).

1.3.3.2. Stromal Cells and their known secreted factors

Although all the NP cells of the liver can be considered its stromal cells, in this section we focus on the cells that are known to play critical roles in the production of ECM within the space of Disse; and cytokines that are believed to affect hepatocytes directly. The two most important stromal cells of the liver can be considered SCs and SECs, being from mesenchymal and endothelial origin respectively. SCs are influenced by factors secreted by SECs, hepatocytes, KCs, other resident immunological cells (35) and themselves (13). Non-activated SCs produce mainly collagen III, IV, laminin,
proteoglycans and some collagen I and FN1 (13, 35, 81, 85). In general, the secretion of collagen type I in the liver is very low and is not only produced by SCs but also by SECs and hepatocytes (64, 81). However, in the fibrotic liver SCs produce high amounts of this collagen (81). In fact SCs, and in part KCs, are in charge of keeping the ECM equilibrium (35), while SECs also contribute to the ECM with the production of collagen type IV, collagen type III, laminin, FN1, undulin, proteoglycans and urokinase type plasminogen activator, that activates latent type TGFβ1 (35, 81, 84).

Table 3: Cytokines produced by the liver NP cells that can contribute to the maintenance of the hepatocyte phenotype.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Cytokine</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Stellate Cells (13, 35)</strong></td>
<td>TGFα</td>
</tr>
<tr>
<td></td>
<td>TGFβ</td>
</tr>
<tr>
<td></td>
<td>EGF</td>
</tr>
<tr>
<td></td>
<td>HGF</td>
</tr>
<tr>
<td></td>
<td>KITLG</td>
</tr>
<tr>
<td></td>
<td>IL6</td>
</tr>
<tr>
<td></td>
<td>Epimorphin</td>
</tr>
<tr>
<td></td>
<td>Pleitrophin</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
</tr>
<tr>
<td><strong>Sinusoidal Endothelial Cells (35)</strong></td>
<td>TNFα</td>
</tr>
<tr>
<td></td>
<td>IL6</td>
</tr>
<tr>
<td></td>
<td>HGF</td>
</tr>
<tr>
<td></td>
<td>IGF-1</td>
</tr>
<tr>
<td></td>
<td>TGFβ</td>
</tr>
<tr>
<td><strong>Kupffer Cells (35)</strong></td>
<td>TNFα</td>
</tr>
<tr>
<td></td>
<td>IL6</td>
</tr>
<tr>
<td></td>
<td>HGF</td>
</tr>
<tr>
<td></td>
<td>TGFβ</td>
</tr>
</tbody>
</table>
As previously mentioned, SCs, SECs, and KCs secrete cytokines that can support the hepatocyte mature phenotype (Table 3). However, the interaction between hepatocytes and these other cell types is far more complex. SECs, SCs, KCs, and hepatocytes all interact with each other significantly affecting their phenotypes, driving them towards either a homeostatic or pathological state (13, 74, 86, 87). For instance, SECs contribute to the maintenance of the SC inactivated phenotype through the secretion of NO (74); and SCs and hepatocytes contribute to the maintenance of the SEC phenotype through the secretion of VEGF (86). Thus, a delicate balance between all liver cells is required in order to maintain their normal phenotype.

1.4. Mesenchymal Stromal Cells (MSCs)

As previously mentioned in sections 1.3.1 and 1.3.2.2, liver pericytes or SCs are of mesenchymal origin. As SCs, MSCs have been shown to be able to play a role as hepatocyte stromal cells: hepatocytes increase their survival and functionality when they are cultured together with MSCs (further explained in section 1.4.2). According to the International Society for Cellular Therapy a MSC is a cell that has to comply with at least 3 criteria: “First, MSC must be plastic-adherent when maintained in standard culture conditions. Second, MSC must express CD105, CD73 and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA-DR surface molecules. Third, MSC must differentiate to osteoblasts, adipocytes and chondroblasts in vitro” (88).

1.4.1. Bone Marrow Mesenchymal Stromal Cells

Bone marrow stromal cells (BM-MSC) are the most common source of MSCs, and are acquired from the adherent population of the bone marrow (BM), as such they are highly heterogeneous even after the elimination of any hematopoietic (CD45+) cells with subsequent passages. It is considered that the BM-MSCs contain the mesenchymal stem and progenitor cells, which are thought to decrease with age due to the lower number of CFU-F reported with age (Figure 7); and it has been suggested that their specific location
within the bone marrow is the perivascular compartment (89, 90). All the marrow stroma, which contains vessels, ECM and various cellular components, is considered to support an adequate niche for haematopoiesis, and thus hematopoietic stem cell maintenance and proliferation (91). However, BM-MSCs seem to be of crucial importance for the formation of the marrow stroma as they as whole, and even some of its sub-colonies, are able to form ectopic bone with a functional bone marrow cavity while being part of both the fibrous and osseous tissue and having host-derived hematopoietic components (90, 92). Muraglia et al (93) found that within human BM-MSCs there were tripotent (osteogeneic (O), chondogeneic (C), adipogeneic (A)), bipotent (O,C), and unipotent (O) populations, and they reported the default lineage as osteogeneic. Nevertheless as they neglected the fibroblastic state of BM-MSCs, it can be argued that when the potential to differentiate into the osteogeneic lineage is lost, there still remains the fibroblastic potential, which despite being often ignored by the MSC researchers, is highly abundant and of crucial importance in the body.

BM-MSCs have been shown to have a strong potential for the treatment of immune diseases due to their immunoregulatory and immuneprivileged phenotype (96, 97); they have been employed to treat many conditions including those associated with cardiac, skin, tendon, neural, liver, bone, cartilage and muscle therapy (91, 96, 98).
1.4.2. Mesenchymal Stromal Cells as Supportive Cells for Hepatocytes

The following was published as mini-review (65), and is reproduced herein without modification and agreeing with Nature’s Publishing Group policies.

1.4.2.1. Abstract

Hepatocytes and hematopoietic stem cells appear to share many of the same requirements for their survival, functionality and proliferation. This may be due to a shared location during foetal development. Moreover, hepatocytes and hematopoietic stem cells are unable to function, or even survive, without stromal cell support. Bone marrow-derived mesenchymal stromal cells support the proliferation and functionality, not only of hematopoietic stem cells, but also of hepatocytes. While knowledge of the mechanisms underlying hematopoietic stem cells support is far more advanced than for hepatocytes, data suggest that many agents important for HSC also maintain the normal hepatocyte phenotype in vitro. Thus, it is possible that new techniques for the maintenance and expansion of hematopoietic stem cells may also be useful for hepatocytes. Bone marrow-derived mesenchymal stromal cells are easily cultured and expanded in vitro, and some data suggest that they are immunoregulatory as well as relatively non-immunogenic. These observations suggest that allogeneic mesenchymal stromal cells may be useful not only in supporting hepatocyte growth and proliferation but also in modulating immune responses such as stellate cell activation.

1.4.2.2. Introduction

All organs in the body comprise a supportive component, the stroma, and a functional component, the parenchyma, and a symbiotic (99, 100) relationship between the two is increasingly recognised (87, 101, 102). In the liver, hepatocytes are supported by the hepatic vascular system and the biliary tree, in addition to the connective tissue septa. Cells from each of these structures have been shown to influence hepatocytes \textit{in vitro},
either through cytokine and/or matrix secretion (87, 103-106) or by direct cell contact communication (87, 107). Similarly, haematopoiesis, which starts in the yolk sac, foetal liver, thymus and spleen, but becomes exclusive to the bone marrow compartment, also depends on a stromal component at all of these locations (108). In the bone marrow, the stroma is similarly composed of marrow vasculature and connective, or reticular tissue. The cells that elaborate the latter, like all connective tissue lineages, are derived from a mesenchymal stem cell. However, in the absence of definitive experimental demonstration of the existence of a mesenchymal stem cell, The International Society for Cellular Therapy has referred to these cells as bone marrow mesenchymal stromal cells (BM-MSC), and described them as culture-adherent; positive for CD105, CD73 and CD90; negative for hematopoietic-specific surface molecules; and which can at least differentiate in vitro into bone, cartilage and fat cells (88). Hematopoietic stem cells (HSC) are unable to either survive, proliferate or develop their full differentiation potential in the absence of mesenchymal stromal cells or a supportive cell population (109, 110). In rare cases the adult liver is still able to support HSC and their progenitors, and thus has the potential to provide HSC requirements (55, 111). This capability to provide stromal support to HSC poses an interesting question: If cells from the liver can support HSC, can cells from the bone marrow support hepatocytes?

Hepatocytes are cells that, like HSC, are unable to function effectively without appropriate environmental cues. When hepatocytes are implanted in vivo, they survive extrahepatically in the spleen and thymus; two sites where foetal haematopoiesis occurs (112, 113). In vitro, hepatocytes in basal medium lose their functionality and die in a matter of days (99, 106, 114). Supplements such as dexamethasone, ascorbic acid, nicotinamide, epidermal growth factor (EGF) are able to maintain these cells for longer periods with a slower loss of their functionality (115, 116). Other attempts to improve in vitro cultures have been reported by also coculturing hepatocytes with a variety of other cell types in an effort to emulate the original in vivo environment (Reviewed by Bhatia et al, 1999 (87)). The cell types used have included endothelial and epithelial cells, NP liver cells, such as liver epithelial cells or stellate cells, fibroblasts and BM-MSC (62, 87, 99, 117-126). Of these, BM-MSC present important advantages: some data suggest that they are immuneprivileged, immunomodulatory and are easily expanded in vitro (127-
Thus, they may serve an additional role in hepatocyte transplantation by diminishing the host allogeneic response. Furthermore, given that BM-MSC can regulate stellate cell activation (130, 131) they may also induce regression of cirrhosis.

1.4.2.3. MSCs support Hepatocyte Function

Corlu et al showed that MSCs are able to preserve hepatocyte morphology longer than occurs in hepatocyte monocultures (107, 132). They also demonstrated that the membrane-associated liver-regulating protein (LRP) is in part responsible for this support; and when antibodies against this protein are added daily to the cocultures, the effect is lost (107). LRP is expressed by BM-MSCs, hepatocytes, liver epithelial cells, stellate cells, Kupffer cells and similar cell types such as those in the thymus and spleen (133). In cocultures of rat hepatocytes, and rat liver epithelial cells, they showed that LRP is essential for maintaining the mature hepatocyte phenotype and that cell contact is required for this effect (134). Interestingly, this protein appears to be implicated in HSC support: when LRP activity of BM-MSC and rat liver epithelial cells is inhibited, HSC supportive capacity is significantly diminished (135).

The capacity of BM-MSCs to support hepatocytes has been further investigated both in vivo and in vitro. In vitro, in addition to many functional characteristics, the morphology of hepatocytes is maintained. Isoda et al, showed that hepatocyte/BM-MSC cocultures were able to keep the albumin and ammonia metabolic capacity at higher levels than in controls (136). This appeared to be independent of cell-cell contact. Both conditioned medium and transwell coculture showed either the same or better maintenance than observed with contact cocultures. They concluded that interleukin 6 (IL6) was one of the factors involved, since urea production was significantly improved when compared with untreated monocultures, or cultures with antibodies against IL6. This factor has a cytoprotective effect which has been demonstrated both in vivo and in-vitro (137, 138). In the presence of harmful agents, hepatocytes exposed to IL6 are able to maintain higher levels of albumin and urea secretion and also exhibit a better capacity to metabolize drugs (137). IL6 has previously been described to have effects on liver regeneration; it can...
induce proliferation but in some cases can cause growth arrest (139, 140). This may be explained by the findings of Sun et al who showed that IL6 alone induces quiescence in hepatocytes, but in the presence of NP cells, the effect is the opposite. IL6 stimulates NP cells to produce hepatocyte growth factor (HGF) which is a strong hepatic mitogen and one way by which it induces hepatocyte proliferation (140).

Mizuguchi et al showed additionally, that cell contact was important for hepatocyte proliferation while the differentiated state was maintained (99). They showed maintenance of the expression of hepatic-specific genes and proteins. One of the important proteins maintained was tryptophan 2,3-dioxygenase, which is downregulated significantly upon hepatocyte dedifferentiation. C/EBPα and C/EBPβ were also present. C/EBPα is a transcription factor that induces the adult hepatocyte phenotype and promotes quiescence (99, 141). Conversely, C/EBPβ is crucial for normal hepatocyte proliferation and response to growth factors (142, 143). It appears that BM-MSC/hepatocyte coculture allows for a balanced coexpression of these two transcription factors, showing proliferation without the loss of the mature phenotype. Hepatocytes in coculture remain polarized and able to form bile canaliculi, characteristics of adult hepatocytes (144).

Additionally, Gu et al showed that hepatocyte/BM-MSC cell proportions, soluble factors, and secreted extracellular matrix (ECM) components were involved in hepatocyte maintenance (145). Unlike others, Gu et al used porcine BM-MSC and hepatocytes, a species which could be potentially used for bioartificial liver systems. They demonstrated that the proportion of 2:1 (hepatocyte/BM-MSC) enabled hepatocytes to exhibit greater albumin and urea synthesis. The investigators also demonstrated that when BM-MSC matrix secretion is impaired using siRNAs, the effect of BM-MSCs on hepatocytes was diminished; albumin and urea production decreased compared to regular contact cocultures. Despite the effect of ECM proteins on the interaction, it was mentioned that in coculture without cell contact, the levels of both albumin and urea syntheses are higher than in monoculture, thus confirming the role of soluble factors on hepatocyte maintenance.
In other configurations, Takeda et al cocultured BM-MSCs and hepatocytes in hydroxyapatite scaffolds (118). They observed higher albumin production levels *in vitro*. In addition, scaffolds, with hepatocytes and BM-MSC, implanted into analbuminemic rats and cirrhotic mice increased serum albumin levels significantly more than did a scaffold with hepatocytes alone. Furthermore, they also detected higher levels of IL6 in the cirrhotic mice implanted with scaffolds with cocultures. This higher level of IL6 in serum was suggested by Takeda et al as one of the factors that could improve the liver function in these models. One aspect that may have been of significance, using BM-MSCs as stroma, is the fact that BM-MSCs are strong angiogenic inducers through the secretion of vascular endothelial growth factor (146, 147). This alone would allow for higher hepatocyte survival in the scaffold and also a higher systemic effect due to the ability of hepatocytes to interact with the host’s bloodstream.

In most of the hepatocyte/BM-MSC coculture literature, it could be argued that BM-MSCs have hepatic potential that can be induced both by growth factors and by coculture, either by direct cell-cell contact or separated by a semipermeable membrane (121, 148-152). This can result in misinterpretation of data when testing the capacity of BM-MSC to support hepatocytes, since higher levels of albumin and urea can be generated through cells differentiating from the heterogeneous BM-MSC population, rather than from the hepatocytes alone. Nevertheless, Lange et al, showed using GFP-labelled BM-MSCs, that hepatocytes maintained their viability in such cocultures in contrast to hepatocyte monocultures (121). In addition, BM-MSC hepatic differentiation likely accounts for only a small percentage of the population, as conditioned medium is able to induce changes similar to those of transwell cocultures. Taken together, these data suggest that any differentiation that occurs may not significantly change the outcome of hepatocyte/BM-MSC coculture experiments (136, 153).

Furthermore, BM-MSC conditioned medium not only maintains hepatocyte functionality, improves hepatocyte survival and proliferation *in vitro* (136, 153) but can also have pronounced effects in vivo, providing significant rescue from fulminant hepatocyte failure (FHF) (153, 154). In a rat model, where FHF was induced with D-galactosamine, the animals were treated with conditioned medium or saline. Treatment with conditioned
medium resulted in reduced apoptotic hepatocellular death and lower inflammatory response-effects that translated into a higher survival rate in the animals (153, 154). These data suggest that BM-MSCs may provide a means to identify active biotherapeutic agents rather than being used directly in conventional cell therapy.

1.4.2.4. Putative Mechanisms for MSC-Hepatocyte Interactions

BM-MSCs appear to provide a number of cues for hepatocyte growth and development (Figure 8). They secrete cytokines important in haematopoiesis that also mediate hepatocyte proliferation and differentiation. HGF enhances haematopoiesis by acting synergistically with other factors (155-157) and is a highly potent mitogen of hepatocytes, induces hepatocyte maturation and has a cytoprotective effect (48, 157, 158). Stem cell factor (KITLG), involved in hematopoietic progenitor cell survival, renewal and differentiation (139, 159) also stimulates hepatocyte proliferation (139, 160). KITLG appears to act downstream of tumour necrosis factor alpha (TNFα) and IL6 hepatocyte proliferative pathways, as both TNFα and IL6 proliferative effects are, in part, due to KITLG (139, 160). Liver NP cell IL6 secretion is stimulated by TNFα, also secreted by BM-MSCs (161). Upon liver damage, TNFα serum levels increase, thereby activating IL6 expression (162). TNFα also has direct effects on hepatocyte proliferation and matrix secretion (163), and also affects HSCs by increasing their proliferation and differentiation rate in the presence of IL3 (164). Epidermal growth factor (EGF) is a potent mitogen of hepatocytes, produced by BM-MSCs at variable levels (48, 163, 165). Transforming growth factor-β (TGFβ), an antimitogenic agent, is secreted by BM-MSC (48). However since these cells adapt to different environments and change their cytokine secretion rate (165), it is possible that the net effects, BM-MSCs have over hepatocytes, are mitogenic. TNFα confers resistance to HSC against TGFβ, which also has antiproliferative effects on HSCs (166), an event that could also happen with hepatocytes, diminishing any antiproliferative effect arising from TGFβ (164, 167).

Another environmental cue that significantly affects hepatocyte functionality is the extracellular matrix (103-105, 145). BM-MSCs in coculture actively synthetize collagen
type I, which can embed hepatocytes to provide an effect similar to a collagen sandwich monoculture (104-106, 144). Collagen gel sandwich cultures of hepatocytes help to preserve hepatocyte function for weeks as opposed to standard monocultures where functionality is lost within the first days of culture (104-106). Other proteins that have effects on hepatocyte functionality and can be secreted by BM-MSC include laminin and fibronectin (103, 145, 168, 169). Of note, laminin is mainly expressed in foetal liver while fibronectin is produced in adult liver (103). Other possible important extracellular components secreted by BM-MSCs include dermatan and chondroitin sulfate-containing proteoglycans. They have been associated with the preservation of cell communication junctions and maintenance of normal levels of hepatocyte-specific genes (170-173).

Figure 8: Putative mechanisms by which MSCs support hepatocytes, as described in the text. Reciprocal mechanisms are not illustrated since they are beyond the scope of this article.

Cell contact also plays an important role on hepatocyte maintenance. At low densities for instance, hepatocyte proliferation is enhanced (174). Direct contact effects have been shown by Corlu et al as an important aspect for the maintenance of hepatocyte morphology (107) and by Mizuguchi et al as a mediator of the polarized state and cell proliferation (99, 144). As mentioned previously, Corlu et al identifies LRP as a key player of cell-cell communication. However, cell-cell communication in this system is more complex. For example, Connexin-43 correlates with the capacity of fat-storing cell clones in maintaining hepatocytes (175). Connexin-43 is not only present in BM-MSC
but may be involved in inter-MSC communication (176). It is conceivable that this gap-
junction protein provides another means for BM-MSCs to communicate with
hepatocytes.

It has been suggested that Jagged1-dependent Notch signalling is involved in BM-
MSC/hepatocyte communication due to an increase of Jagged1 expression in BM-MSC
when cocultured in direct contact with hepatocytes (99). Notch signalling is a conserved
evolutionary mechanism initiated by a ligand–receptor interaction between neighbouring
cells (58, 177, 178). It regulates a broad range of events during embryonic and post-natal
development, including proliferation, apoptosis, border formation, and cell fate decisions
(177). Notch signalling is involved in the development of vertebrate self-renewing
organs, during tumourogenesis, in the inhibition of differentiation, in lineage
specification at developmental branch points and in the induction of differentiation (58,
177, 179-181). More specifically, the Jagged1-Notch interaction is implicated in cell fate
decisions in hepatoblasts and promotes the formation of haematopoietic primitive
precursor cell populations (58, 178, 182, 183). Furthermore, downregulation of Notch
signalling promotes the development of the mature hepatocyte phenotype in hepatoblasts
(58), suggesting that its upregulation might be related to a controlled dedifferentiation
that leads to proliferation.

HSCs and progenitors form cell-cell contacts with BM-MSCs in vivo (178). In vitro, such
contacts appear essential for the maintenance of long-term HSC cultures (178). Stromal
cells from both foetal liver and bone marrow express Jagged1 (182); in addition,
hepatocytes express Notch1 both in vivo and in vitro (184); and in the regenerating liver
the levels of Jagged1 and Notch1 are upregulated (185). This evidence further supports
the possibility that BM-MSCs interact with hepatocytes by the Jagged1-Notch1
mechanism.

BM-MSCs are capable of differentiating into multiple lineages in vitro and in vivo to
bone, cartilage, marrow stroma and adipose cells (168). It is unclear therefore whether
BM-MSCs found in vivo or a type of differentiated BM-MSC is involved in hepatocyte
maintenance. Mizuguchi et al. suggests that BM-MSCs, when supporting hepatocytes, are
mainly fibroblasts due to their high production of collagen type I (144). Given that BM-MSCs have the capability of differentiating into other cell types, it is possible that an adipocytic or pre-adipocytic cell provides the support required by hepatocytes, especially because of data implicating fat storing cells in mediating this nurturing process (125, 175). It can, however be argued that epithelial cells such as rat liver epithelial cells (RLEC) are able to provide support to hepatocytes. It should be noted however, that BM-MSCs are very similar to the pericyte population (186), hence the stellate cell may be the closest type anatomically to support hepatocytes, given that it is both lipocytic and pericytic (72).

1.4.2.5. Other Sources of MSCs

Recent studies show that mesenchymal stromal cells share characteristics that are common regardless of the tissue source (187-189). In conducting in vitro studies it is worthwhile to explore alternative sources of MSCs as a potential support for hepatocytes. For example, adipose-derived mesenchymal progenitors are easily acquired in great quantities although like BM-MSCs, their harvesting is invasive (190). Moreover, they do not support haematopoiesis as efficiently as do BM-MSCs (191, 192). In contrast, placenta and umbilical cord-derived MSCs are obtained non-invasively, support haematopoiesis (193-195) and data suggest that they have a similar non-immunogeneic and immunomodulatory phenotype to BM-MSCs (196, 197). Further studies are required with these easily accessible alternatives to assess their ability to support hepatocytes in vitro. Studies of the co-culture of MSCs with hepatocytes have uncovered important mechanisms that mediate the maintenance, proliferation and differentiation of hepatocytes and may eventually lead to their manufacture for clinical use.

1.4.1. Other source of Mesenchymal Stromal Cells

This section is an expansion of the previous, 1.4.2.5, and was kept separate in order to preserve the fidelity of the published text presented in section 1.4.2.
As previously mentioned, adipose tissue is considered to be a promising alternative source of MSCs as it is normally discarded after liposuctions and the colony forming unit-fibroblast frequency (CFU-F) at harvest is 1:1795 (188). They are able to differentiate into at least the three mesenchymal lineages (O, C, A) and express CD105, CD73, CD90, CD146 and lack the expression of CD14, CD45 and MHCII. Interestingly, among their population there is still a considerable number of CD34+, varying up to around 28% (188, 198).

MSCs, showing trilineage differentiation capacity and complying with the expression of CD105, CD73 and CD90, and the lack of MHCII, have also been harvested from umbilical cord blood. However, from the total mononuclear population the CFU-F frequency is only $1:5 \times 10^8$ (188).

Foetal tissues such as foetal bone marrow, liver and blood have likewise been shown to contain mesenchymal progenitors that may have all the characteristics of MSCs as they are at least CD45- and able to exhibit trilineage differentiation. It is worthwhile noting that the CFU-F at harvest of the first trimester blood was found to be $1:1.2 \times 10^5$, $1:8.85 \times 10^4$ for the liver and $1:7.94 \times 10^4$ for bone marrow (199).

Currently MSCs have been found in many organs and tissues very possibly due to the fact that data suggest that MSCs exist within the pericyte population (186, 200). Crisan et al. 2008 (186), using the pericyte marker CD146 and the endothelial marker CD34, showed that cells isolated from skeletal muscle, pancreas, adipose tissue, placenta and bone marrow, enriched for CD146$^\text{high}$CD34-, express αSMA, NG2, CD90, CD73, CD105, PDGF and no HLA-DR, endothelial or hematopoietic markers (CD144-, CD31- and CD45-). In addition it was also shown that these sorted cells were able to differentiate into the osteogeneic, chondrogenic and adipogeneic lineages.

While it appears that MSCs can be found everywhere, the important issues are:

1) Realistically, marrow and adipose tissue are the only two tissue sources that are currently being used to harvest MSCs that can be expanded to clinically relevant numbers.
2) For allogeneic MSC treatments, marrow and adipose tissue rely on harvesting tissue from adult donors due to the fact that it is not ethically viable or even practical to harvest from individuals below the age of consent.

3) Placenta and umbilical cord represent a neonatal source of MSCs from tissue normally discarded at birth i.e. medical waste (201-203).

4) Neonatal MSCs are more proliferative and thus allow for a faster acquisition of relevant numbers for any cell therapy (201, 202).

Thus, MSCs from neonatal tissues hold a promising potential, as they are easily acquired and their availability is potentially unlimited for allogeneic uses. One of the neonatal MSCs, acquired from within the Wharton’s jelly, specifically from the perivascular area of the umbilical cord is hence described more thoroughly below.

### 1.4.2. Human Umbilical Cord Perivascular cells

Human umbilical cord perivascular cells (HUCPVC) are cells that are acquired from the Wharton’s Jelly (Figure 9) surrounding the umbilical cord vessels. They express makers used to identify pericytes: \(\alpha\)SMA, NG2, 3G5 and CD146 (202, 203). Interestingly, a similar expression of NG2 to hBM-MSCs is present but they have a significantly higher expression of CD146 Figure 10) (202). To date, these cells have shown many similarities with BM-MSCs (Figure 10) while exhibiting a higher proliferation rate (202) and CFU-F at harvest — being 1:333 (203) compared to 1:10^4 for hBM-MSCs in a newborn (94) (Figure 7). HUCPVCs express CD73, CD90 and CD105, and lack the expression of CD45, CD34, MHCII (203), and are also capable of at least differentiating into the osteogenic, chondrogenic and adipogenic lineage (202, 204). According to Sarugaser et al (204), HUCPVCs are a heterogeneous population containing cells with different differentiation potential: pentapotent (Myogeneic (M), O, C, A, Fibroblastic (F)), tetrapotent (O, C, A, F), tripotent
(O, C, F or O, A, F), bipotent (O, F or C, F) and unipotent (F). As cells would age they could lose the myogenic, chondrogenic, adipogenic and osteogenic capability. O and F cells were able to self-renew and were more common; followed by the chondrogenic potential (C, F) which were not shown to self-renew. The default lineage was that of a committed fibroblast, which could still renew itself. When these results are compared to those previously presented by Muraglia et al (93) (described in section 1.4.1); it can be found that after the fibrogenic capacity, neglected by Muraglia et al, the osteogenic capacity is the most persistent of the potentials in MSCs.

HUCPVCs, as hBM-MSCs, are both non-alloreactive and immunoregulatory in vitro (196). To date, they have been shown to contribute to skin (205), tendon (206), bone and cartilage wound healing (204). They have also shown potential for neural regeneration (207) but their true potential is still unknown as they are a relatively new MSC population.

Figure 10: Representative flow cytometry histograms of HUCPVCs and BM-MSCs. Flow cytometry data for one cryopreserved cord sample at passage 2, one plated cord sample at passage 1, and one cryopreserved BM-MSC sample at passage 2. The black histogram is the cell staining of the negative control. The red histogram is the antibody-stained cells. Legend and picture taken from Matta, R 2009 (208).
1.5. Hepatocyte Cultures

End-stage liver disease is usually treated by liver transplantation, however donor organ shortages remain a serious problem, as previously mentioned in section 1.2. Due to the regenerative potential of the liver, a temporary support with hepatic supplementation systems, i.e. BALS, would make many transplantations unnecessary (15, 16). Because of the main role of hepatocytes in the liver, their culture represents a model for drug testing/toxicology and a source of cells for therapy and hepatic supplementation (19, 20). However, despite their remarkable regenerative ability in vivo, the therapeutic potential of cultured hepatocytes is restricted as their proliferation is minimal post-extraction; and they soon start losing their functionality and die (15, 19, 99).

Hepatocyte culture only started after finding a viable technique to isolate them. Since 1952 there was a search for the development of such technique, a technique that would allow for the isolation of single hepatocytes without their destruction. Initially mechanical methods combined with EDTA or citrate perfusion were tried but it was not until the use of collagenase that high quality single hepatocytes were acquired (209). Howard and coworkers first isolated hepatocytes using collagenase in 1967 (209); later in 1969 Berry and Friend (210) developed the intact organ perfusion with collagenase and significantly increased the hepatocyte yield, thus leading to the current standardized technique of hepatocyte and NP cell isolation.

Primary hepatocyte cultures can be established by taking an hepatocyte cell suspension and placing it, as with other cell types, on tissue culture treated surfaces or on protein coated surfaces, i.e. collagen, fibronectin, laminin. Usually in standard culture conditions using medium supplemented with at least serum, insulin, and corticosterone, hepatocytes show a significant decrease in viability within the first 48 hours, and continue to senesce thereafter (106, 211), when they start showing changes in their original morphology, becoming less polygonal and more spindle-shaped and also more translucent, being less granular; these changes get to be more apparent as the time of culture increases. Other phenotypic changes include the alteration of hepatic enzymes such as pyruvate kinase I, glucokinase, aldolase B and phosphoenol pyruvate carboxykinase, changes in
cytochrome P450 protein levels, urea and glycogen synthetizing enzymes and loss of ascorbic acid and insulin receptors. Also, hepatocytes in culture gradually increase their oxygen consumption and eventually may express α-fetoprotein (AFP), a protein only produced by hepatoblasts or foetal hepatocytes (211). In addition within the first week of culture, hepatocytes decrease their albumin secretion to less than one tenth of their secretion levels on day 1 (106).

### 1.5.1. Hepatocyte Monoculture

As mentioned above, hepatocyte monoculture is usually not able to allow the maintenance of the hepatocyte phenotype, thus it has been the aim of intensive research to find ways to both support mature hepatocytes for long or longer periods of time. One of such ways is by the addition of soluble factors to the medium; the addition of ascorbic acid to the medium can restore the normal intracellular concentration of this factor in hepatocytes, increasing the concentration of cytochrome P450 proteins (212). Insulin and dexamethasone can act independently and synergistically, maintaining at least the secretion of albumin (213), and only when combined with glucagon allow for the maintenance of the urea synthesis (214). DMSO, at 0.5-2%, improves the maintenance of hepatocyte viability, their morphology, and their albumin secretion capability, while diminishing the growth of NP cells (215). Nicotinamide can maintain hepatocyte viability, improve the maintenance of albumin and tryptophan 2,3-dioxygenase expression (216) and can also act synergistically with EGF in inducing hepatocyte proliferation (115).

It has been seen that in positively charged surfaces or proteoglycan coated surfaces, hepatocytes do not form cell-sheets but aggregate forming hepatocyte spheroids which improve the maintenance of the hepatocyte phenotype, probably due to the increase in cell interactions (217). Matrigel, a basement membrane protein mix extracted from Engelbreth-Holm-Swarm tumours (218), can induce spheroid formation (219, 220). It is composed of 90% of laminin, 6% collagen type IV and 4% heparin sulphate proteoglycan. Matrigel has shown to induce the maintenance of albumin secretion, cytochrome P450 protein levels and the ultrastructure integrity, including cuboidal
morphology and granularity of hepatocytes. Interestingly, none of Matrigel’s components on their own are able to support albumin secretion by hepatocytes (218).

The collagen (type I) gel sandwich system provides a beneficial environment to hepatocytes; the benefit provided might be due to the fact that this technique gets one step closer to the in vivo environment where hepatocyte cords are in direct contact and surrounded by ECM. In this method, hepatocytes are first seeded on top of a collagen gel and after being attached another layer of collagen is laid on top of them creating the so-called collagen sandwich. In 1989, Dunn et al (106) demonstrated that rat hepatocytes, when cultured using this technique, were able to maintain the original morphology and the albumin secretion capacity for a period of at least 7 weeks; later it was shown that this technique also improved the maintenance of the urea synthetic ability compared to monoculture despite its reduction to 20-30% of the first day levels after 10 days (221). Furthermore, hepatocytes that had a degraded phenotypic stage (more than one week on a single layer of collagen) were able to recover their albumin secretion capacity when covered by a second layer of collagen (106). Later Kono et al (105), confirmed these observations in human hepatocytes and also showed improved maintenance of the CYP1A activity.

As shown by Moghe et al (222), both Matrigel and collagen sandwich configurations provide comparable maintenance of albumin secretion by hepatocytes, making them the two most successful monoculture techniques, but each of the systems induces different expression patterns of membrane proteins. In Matrigel, hepatocytes express the membrane proteins F-actin, E-cadherin, glucose transporter II, the Na+K+ATPase, Connexin-32, aminopeptidase N, dipeptidyl peptidase IV and the EGF-receptor. They are present in their respective places in normal hepatocytes but most of them are also present in the cell-matrix contact domain (corresponding to the sinusoidal domain in the liver), with the exception of Connexin-32, where only the EGF-receptor should be localized. On the other hand, hepatocytes in collagen sandwich cultures express most of the proteins in the corresponding regions where they appear in the normal liver but these hepatocytes lack the expression of both Connexin-32 and the EGF-receptor. Another important comparison between Matrigel and the collagen sandwich culture was by Boost et al
(223), where they looked at the improvements related to the production of coagulation factors, which is an important function of hepatocytes and one that can aid patients with coagulation factor disorders. They observed that hepatocytes when cultured using the collagen sandwich method showed significant improvement in the secretion and expression of coagulation factors over time, especially antithrombin III, factor VII and VIII.

Despite the success of Matrigel and collagen sandwich culture, hepatocytes still loose some of their normal phenotype, as can be seen by the loss and relocalization of their membrane proteins (see above). In addition, the BLSS BALS (Table 2) which uses collagen entrapped hepatocytes fails to presently provide supplementation for longer than 12 hours indicating that the current methods in monoculture are not enough for improving the lifespan of BALSs. Thus, the coculture of hepatocytes with cells that can serve as stroma may provide the missing factors for the improvement of hepatocyte cultures.

### 1.5.2. Hepatocyte coculture

Several attempts to improve hepatocyte culture have been reported by coculturing hepatocytes with a variety of other cells types in many different proportions, going from 1:14 to 14:1 (77, 87). The cell types used include NP liver cells, fibroblasts, other epithelial cells and BM-MSCs (Table 4). The effects of these cocultures on hepatocytes have shown an improved maintenance of their functionality and in some cases enhanced proliferation (87, 99, 117-121).

In 1979, Langenbach et al first noticed that hepatocytes cultured on an irradiated feeder layer of fibroblasts significantly improved their viability and metabolic capacity compared to monocultures (87, 224). Thus, it was discovered that cells of mesenchymal origin were able to serve as stroma, and later RLECs also demonstrated stromal capability, showing that this property was not confined to the mesenchymal lineage.
Goulet et al (225), compared the effect of 6 putative stromal cells on hepatocyte albumin secretion and morphology. All of the cocultured hepatocyte cultures regardless of the cell type used as stromal cells, maintained a healthy morphology compared to monoculture, where hepatocytes turned flat, lost their granularity and died within 5-6 days. All the cocultured hepatocytes maintained a better albumin secretion than hepatocyte monoculture, but there were obvious differences in the levels: RLECs and mouse embryonic fibroblasts provided the highest enhancement to hepatocyte albumin secretion.

Bhandari et al. (114) showed that 3T3 murine fibroblasts when used as stromal cells for hepatocytes, improved their survival, maintenance of their albumin secretion and cytochrome activity. Furthermore, fixed fibroblasts were also able to improve albumin secretion after 8 days of culture, showing a delayed effect of factors that require cell contact. On the other hand, fibroblast conditioned medium was able to show improvements of albumin secretion the earliest, but it never increased the secretion as much as live or fixed fibroblasts. Thus, the ways in which fibroblast affect hepatocyte not only involve soluble factors but also either or both ECM and contact interactions.

Table 4: Cells used as hepatocyte stromal cells. Table based on reference (87).

<table>
<thead>
<tr>
<th>Liver-derived</th>
<th>Non-liver derived</th>
</tr>
</thead>
<tbody>
<tr>
<td>RLEC (biliary) (226, 227)</td>
<td>Bovine aortic endothelial cell (225)</td>
</tr>
<tr>
<td>SC (62, 63)</td>
<td>Canine kidney epithelial cell</td>
</tr>
<tr>
<td>SEC (73, 225)</td>
<td>Chinese hamster epithelial cell</td>
</tr>
<tr>
<td>KC (68)</td>
<td>Embryonic murine fibroblast (114, 225, 228)</td>
</tr>
<tr>
<td>Liver NP cell (123, 229)</td>
<td>Human fibroblast (225)</td>
</tr>
<tr>
<td>Human Cholangiocyte (77)</td>
<td>Human lung epithelial cell</td>
</tr>
<tr>
<td></td>
<td>Human venous endothelial cell</td>
</tr>
<tr>
<td></td>
<td>Monkey kidney epithelial cell</td>
</tr>
<tr>
<td></td>
<td>Rat dermal fibroblast (225)</td>
</tr>
<tr>
<td></td>
<td>BM-MSC (rat (99, 118, 119), pig (230))</td>
</tr>
<tr>
<td></td>
<td>Whole Bone Marrow (231)</td>
</tr>
</tbody>
</table>
Donato et al (232) also showed that murine fibroblast are able to maintain hepatocyte cytochrome activity better than conventional monoculture but they also proved that cocultures were better than monocultures in Matrigel (described in 1.5.1). Furthermore, the two fibroblast cell lines used were shown to affect hepatocytes to different degrees, one being slightly better than the other. Khetani et al (228) showed that three different murine fibroblast cell lines had different levels of support on hepatocytes, showing a high, medium and low supportive capability based on albumin secretion and urea synthesis by the cocultured hepatocytes. This proved that not all murine fibroblasts or all cells from a cell type have the same supportive capability; and that there is a population with specific characteristics that is better suited to be a putative hepatocyte stromal cell. After performing microarray analysis on the three different cell lines they found factors that correlated negatively and positively with the capacity to support hepatocytes. Among them they tested decorin, seeing that it was able to provide an improvement in hepatocyte ureagenesis and albumin secretion and to significantly improve the supportive capacity of the poorly supportive fibroblasts.

RLECs were able to maintain the albumin secretion capability of hepatocytes and rescue them even after a week in monoculture conditions, thus showing the reversibility of hepatocyte de-differentiation. Sakai et al (227), further investigated the effect of RLECs and compared their effect on two well known improved hepatocyte monoculture techniques: collagen sandwich system (described in 1.5.1) and spheroids. They not only confirmed an improved maintenance of albumin secretion, they also showed that the standard 2D coculture of RLEC and hepatocytes can have similar or even improved effect on hepatocytes compared to spheroids as analysed — not only by albumin secretion but also cytochrome P450 activity. However, the standard RLEC-hepatocyte coculture was not able to provide an enhancement remotely similar to the maintenance capacity of the collagen sandwich system on hepatocytes. Yet, the addition of RLECs to the collagen sandwich system provided a slight increase in the hepatocyte functions assayed (albumin and cytochrome activity), thus indicating that cocultures are an important factor to create improved hepatocyte function. Nevertheless, the latest improvements in hepatocyte monocultures should not be discarded but rather combined
in order to eventually reach a state similar to the one in vivo, where there is not only maintenance of functionality but also a remarkable regenerative capacity.

1.6. Rationale

HUCPVCs provide an easily harvested population of MSCs that proliferate rapidly in culture and exhibit a very high CFU-F frequency (~1:300 isolated cells at harvest), which is significantly higher than neonatal bone marrow (1:10⁴) (233), and umbilical cord blood (1:5x10⁸) (188). Thus, we have a rich population of MSCs which has a similar phenotype to BM-MSCs, including differentiation, and immunomodulatory characteristics. Results to date indicate that MSCs from bone marrow (BM), foetal liver cells and HUCPVCs, as well as other sources, do not cause a response from a mismatched immune system (196, 234-236). However contrary to most BM-MSCs, HUCPVCs express cytokeratins, CK8, CK18 and CK19 (Appendix 2), making them a population of cells with mixed epithelial and mesodermal features, similar to foetal liver stroma (59, 237). An emerging area of mesenchymal research involves the study of BM-MSCs acting as a functional stromal support system for cultured hepatocytes (87, 99, 117-121). Due to the significant similarities between BM-MSCs and HUCPVCs, and indications that HUCPVCs may be closer to foetal liver cells than BM-MSCs, we have an obvious opportunity to evaluate the ability of these cells to provide stromal support for hepatocytes while still keeping the coculture system being non-alloreactive and with anti-inflammatory capacity, properties which may be of importance to further aid in the recovery of patients with liver failure.

1.7. Hypothesis

HUCPVCs are putative stromal cells for hepatocytes and are able to support them through soluble and non-soluble factors, improving their functionality, polarity, morphology and net proliferation.
2. CHAPTER 2: Human Perivascular Cells Support Hepatocyte Function

*The author of this thesis performed all the work presented within this chapter.*
Abstract

Hepatocyte functionality and survival decrease rapidly in culture and both can be improved using bone marrow derived mesenchymal stromal cells (MSCs). We have previously described an alternative, more plentiful, source of MSCs coming from the perivascular area of the umbilical cord, human umbilical cord perivascular cells (HUCPVCs). Our objective was therefore to ascertain whether HUCPVCs could serve as hepatocyte stromal cells ex vivo. For this purpose, rat hepatocytes were cocultured in contact with HUCPVCs (contact coculture). Also HUCPVCs were cocultured separated from hepatocytes with a semipermeable membrane (non-contact coculture) to assess soluble factor interactions. Next, HUCPVC conditioned medium (CM) was used to investigate the possibility of HUCPVC-free support, while flash frozen HUCPVCs were employed to investigate the effects of non-soluble interactions. In all experiments, medium samples were taken daily to assess the production of albumin. Also, at certain days, the levels of cytochrome P450 (CYP) activity and urea secretion were tested. RNA extraction was performed at the end of experiments. Our results show that HUCPVCs in contact and non-contact cocultures with hepatocytes improve albumin gene expression and secretion compared to monoculture. Flash frozen HUCPVCs had a late improvement in albumin secretion while CM improved it for a short period. Urea synthesis maintenance was improved by contact coculture and flash frozen HUCPVCs. CYP activity was significantly increased in the presence of flash frozen HUCPVCs and in non-contact cocultures. We conclude that HUCPVCs can act as stromal cells for hepatocytes, and that soluble and non-soluble factors induce differential effects on hepatocytes.
2.1. Introduction

End-stage liver disease is usually treated by liver transplantation. However, donor organ shortages preclude transplantation for patients in need; only 30% of patients on waiting lists receive a transplant while others are ineligible (14). Also, many patients with acute liver failure die while waiting for a transplant and those with chronic disease often deteriorate so much that they suffer a high mortality rate after transplantation. Due to the regenerative potential of the liver, a temporary support with hepatic supplementation systems, i.e. bioartificial liver systems (BALS), would make many transplantations unnecessary or decrease deterioration while bridging for transplantation (14-17). Because hepatocytes are the main functional cell of the liver, their culture represents a model for drug testing/toxicology and a source of cells for therapy and hepatic supplementation (19, 20). In vivo hepatocytes require the support of the liver stroma, for their functionality and regeneration (15, 19, 59), which contains all the non-parenchymal cells with which hepatocytes interact. Thus, hepatocytes in an ex-vivo environment rapidly lose their functionality (15, 19, 99). For this reason several attempts have been made to provide a more appropriate ex vivo environment for hepatocytes with the objective of replacing what the stroma provides in vivo. One technique has been the coculture of hepatocytes with a variety of other cells types including non-parenchymal liver cells, fibroblasts, other epithelial cells and bone marrow mesenchymal stromal cells (BM-MSC) (87). These cocultures have shown an improvement in the maintenance of hepatocyte functionality, and in some cases enhanced proliferation (87, 99, 117-121). BM-MSCs, particularly, have been shown to improve transcription factor and enzyme expression, and also increase proliferation, and the production of albumin and urea (65). The ways in which BM-MSCs interact with hepatocytes seem to be due to three main factors: cell-cell contact interactions, secreted cytokines and extracellular matrix (ECM) (65).

According to the International Society for Cellular Therapy (ISCT) a MSC is a cell that is plastic adherent, able to differentiate into at least the osteogeneic, chondrogeneic and adipogeneic lineages and expresses CD105, CD73 and CD90 while expressing no hematopoietic markers or HLA-DR molecules (88). BM-MSCs, specifically, are also
immuneprivileged and immunomodulatory (234-236), presenting a further benefit for BALSs since liver failure often results from an uncontrolled inflammatory response within the liver (1). However, the availability of BM-MSCs for BALS applications is limited: the harvest of BM from volunteer donors is an invasive procedure, and MSCs can only be acquired from individuals above the age of consent. Furthermore, the frequency of colony forming unit-fibroblasts (CFU-F) from BM, and the life span of BM-MSCs, decline with increasing age (188, 238), therefore it is expedient to seek alternative, high yield, sources of MSCs.

We harvest human mesenchymal cells from the Wharton’s Jelly surrounding the umbilical cord vessels and call them Human Umbilical Cord PeriVascular Cells (HUCPVC). These cells have shown many similarities with BM-MSCs, and comply with the minimal criteria set by ISCT (202-204), although they exhibit a higher proliferation rate (202) and CFU-F at harvest — being 1:333 (203) compared to 1:10^4 for BM-MSCs in a newborn (94). In addition, as with BM-MSCs, HUCPVCs are both non-alloreactive and immunoregulatory in vitro (196).

Thus, we explore herein the potential of these cells to enhance the maintenance and function of hepatocytes ex vivo. Our results clearly show that HUCPVCs are putative stromal cells for hepatocytes and that their soluble and insoluble factors play different roles in the maintenance of hepatocyte function.
2.2. Materials & Methods

2.2.1. Hepatocyte Isolation and culture

Hepatocytes were isolated from male Wistar rats (Charles River Laboratories, Canada) (250-350 g) by two-step collagenase perfusion of the liver as described previously by Moldeus et al. 1978 (239). The cells were further purified by Percoll centrifugation (10 min at 50 x g; final density of approximately 1.06 g/ml) (240). For each experiment 3 rat livers were harvested. The hepatocytes acquired were plated at a concentration of 28.3x10^3 hepatocytes/cm^2, and maintained in hepatocyte culture medium (HCM): DMEM (Sigma, D6046) supplemented with 10% FBS, 1 µM Dexamethasone (Sigma, D8893), 1x ITS (MP Biomedicals, 2001344), 100 U/ml Penicillin and 100 µg/ml Streptomycin (Invitrogen, 15140-122).

2.2.2. HUCPVC culture and source

HUCPVCs were provided by Tissue Regeneration Therapeutic Inc. The cells were expanded in α-MEM (Invitrogen, 12571-071) supplemented with 10% FBS and antibiotics: penicillin G 167 U/mL, amphotericin B 0.3 mg/mL, gentamicin 50 mg/mL (Sigma). Medium was changed 2-3 times a week, and cells were passaged when confluence was close to 80%. For coculture experiments, HUCPVCs were pooled from at least 5 different donors.

2.2.3. Contact Coculture

The day following the seeding of hepatocytes, after media samples were collected, HUCPVCs were laid on top of hepatocytes at a concentration of 1x10^4 cells/cm^2. For contact coculture and monoculture in 3D configuration, tissue culture treated polystyrene scaffolds (3D Biotek, PS30401) with a fibre diameter of ~300 µm and a separation between fibres of ~400 µm were seeded with hepatocytes according to the manufacturer’s instructions. Briefly, 100 µl of medium containing 672x10^3 hepatocytes was laid on top
of each scaffold and allowed to distribute through the entire scaffold for 3 hours at 37°C in an atmosphere of 5% CO₂. After this time, 400 µl of medium was added. The next day scaffolds were moved to another non-tissue culture treated 24 well plate. For monoculture, 300 µl of medium alone was added, and for cocultures, 300 µl of medium plus 393x10³ HUCPVCs was added. The scaffolds were then left incubating at 37°C in 5% CO₂. During incubation, every 30 min the media was gently mixed 3 times. After 2 hours of incubation, 200 µl of medium were added to each well. The following day all the scaffolds were transferred to a new plate to separate the scaffolds from cells remaining at the bottom of the wells. Media samples were collected daily for albumin measurements and stored at -20°C. The medium used for both cocultures and monocultures was HCM, described above.

2.2.4. Transwell Coculture

The day following the seeding of hepatocytes, after media samples were collected, 24 well-plate 0.45 µm pore size transwell inserts (BD Falcon, 353095) with 20x10³ HUCPVCs were added. Following this, media samples were collected daily for albumin measurements and stored at -20°C. The medium used for both cocultures and monocultures was HCM, described above.

2.2.5. Production of HUCPVC Conditioned Medium

HUCPVCs were seeded at a concentration of 1x10⁴ cells/cm² and were grown for at least 5 days in HCM. To produce the conditioned medium (CM), the medium was changed to DMEM (Sigma, D6046) supplemented with 0.05% bovine serum albumin to prevent protein aggregation. After 20 hours, CM was harvested and concentrated 25X using ultrafiltration units (Millipore, UFC900308) with a 3 kDa molecular weight cut-off. The concentrated CM was stored at -80°C until use.

Hepatocytes were provided with CM at 2 concentrations 1X and 5X: the 25X CM was thawed at room temperature and then was diluted at the appropriate proportions with the
components of the HCM, so that the final medium would be HCM supplemented with 1X or 5X CM medium.

### 2.2.6. Flash Frozen HUCPVCs

After culturing HUCPVCs in HCM for at least 6 days, the medium was removed and the bottom of the plates were immediately submerged in liquid nitrogen until the nitrogen stopped boiling. After flash freezing the plates, they were left at room temperature for 5 minutes and were then transferred to a -80°C freezer over night. The day after, the plates were taken out of the freezer and left at room temperature for approximately 5 minutes, followed by storage at -20°C until use.

The presence of viable cells in the flash frozen HUCPVCs was tested by using the fluorescein diacetate assay: medium with 25 µg/ml of fluorescein diacetate was added to the flash frozen HUCPVCs. After incubating for 10 minutes at 37°C, the wells were washed with medium 3 times and then they were left in medium and visualized with an inverted fluorescence microscope (Olympus IX81 with a Photometrics CoolSnap HQ2 camera).

The day when hepatocytes were seeded on top of the flash frozen HUCPVCs, the plates were first allowed to warm to room temperature before seeding the cells. After seeding, the plates were treated as the other cultures: media samples were collected daily for albumin measurements and stored at -20°C.

### 2.2.7. Mitomycin treatment

To test the effect of HUCPVC number on hepatocytes, HUCPVCs were growth arrested. Briefly, HUCPVCs in culture were incubated in culture medium supplemented with 15 µg/ml of mitomycin C (Sigma, M4287) for 2 hours. Following the incubation, the mitomycin C supplemented medium was removed and the cells were washed 3 times with PBS before adding fresh medium. HUCPVCs were used at least 5 hours after treatment and seeded as described previously. Mitomycin treated HUCPVCs (MT-
HUCPVC) were used at either high (2.5x10^4 MT-HUCPVC/cm² - MH) or low (1x10^4 MT-HUCPVC/cm² - ML) number on hepatocytes, and compared with normal HUCPVCs at an initial seeding density of 1x10^4 HUCPVC/cm².

2.2.8. Hepatocyte Functional Assays:

2.2.8.1. Albumin ELISA

Daily samples stored at -20°C were tested for albumin levels by means of a rat albumin ELISA (Bethyl laboratories, E110-125). Briefly, samples were diluted so that they were within the detection range; MaxiSorp plates (NUNC, 439454) were coated for 1 hour with sheep anti-rat albumin antibody, followed by a 30 min blocking and incubation for 1 hour with the samples. After washing of the samples, the plates were allowed to interact with a horseradish peroxidase-bound Sheep anti-rat albumin antibody for 1 hour for later detection with TMB substrate (Bethyl labs, E102). Finally, sample absorbance at 450 nm was recorded.

2.2.8.2. Urea secretion and cytochrome P450 (CYP) activity measurements

At some time points both cocultures and monocultures were incubated at 37°C with 5% CO₂ for 1 hour while being rocked (200 µl for 24 well plate and 300 µl for scaffolds) in serum-free HCM supplemented with 1 mM ammonium chloride (Stock solution 400 µM) and 5 µM Vivid BOMR substrate (Stock 2mM in acetonitrile, Invitrogen, P2865). Following incubation, 100 µl samples were transferred to 96 well plates and fluorescence (excitation 530, emission 605) was read at 37°C to detect CYP activity. Vivid BOMR substrate can be metabolized by CYP3A and CYP1A family members into a highly fluorescent metabolite. Samples were then frozen at -20°C for later measurement of urea levels. Urea was quantified using QuantiChrom Urea Assay Kit (Bioassay systems, DIUR-500). After diluting the samples to a concentration within the detection range, they
were allowed to react with the working reagent contained in the kit for 50 min and then the absorbance was read at 430 nm.

2.2.9. RNA Isolation and Reverse Transcription

In order to isolate the total RNA from the monocultures and cocultures, they were lysed using Tri Reagent (Sigma, T9424), followed by the company’s recommended procedure. RNA content and quality was measured using spectrophotometry quantitation (NanoDrop 1000, Thermo Fisher Scientific) and genomic DNA elimination and reverse transcription (RT) was performed using the QuantiTect Reverse Transcription Kit (Qiagen, 205311) according to the manufacturer’s instructions.

2.2.10. RT-PCR

End-point RT-PCR was carried out on cDNA resulting from RT, using Platinum TAQ DNA polymerase (Invitrogen, 11668019), according to the manufacturer’s instructions. The annealing temperature used was always 60°C. The amplified PCR product was fractionated on a 2% agarose gel and visualized by SYBR green staining. The primers used are listed in Table 5, all of which were tested for no cross-reactivity with their rat counterparts.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>hACTB</td>
<td>CAGAGCCTGCCTTGGCCA</td>
<td>ACATGCCGGAGCGTTGCG</td>
<td>103</td>
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<tr>
<td>hJAG1</td>
<td>CTTTGGACACTATGGCTGGACC</td>
<td>TACAAGTCCCCCGTGAGACACG</td>
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<tr>
<td>hIL6</td>
<td>GCTATGAACTCCCTCCCACAAGC</td>
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<tr>
<td>hTNFα</td>
<td>ACAAGGCTGAGCCGATTT</td>
<td>TTGATGGCAGAGAGGGTT</td>
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</tr>
<tr>
<td>hEGF</td>
<td>ACCTGAAATGGTGGGAACG</td>
<td>AGGACATCCCATCTCAGTG</td>
<td>220</td>
</tr>
<tr>
<td>hDCN</td>
<td>GCTCTCTACATCCCGCATG</td>
<td>GAAACTCAATCCCAACTTAGCC</td>
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</tr>
<tr>
<td>hGJA1</td>
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<td>ACTGACAGGCCACACCTCC</td>
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<tr>
<td>hKITLG</td>
<td>GTTTGATAAGCCGATGGTGT</td>
<td>GGGTTCTGGCTCCTGAGATG</td>
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<tr>
<td>hLAMB1</td>
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<td>CCGATCTCTGAAAGGTTCCTCC</td>
<td>196</td>
</tr>
<tr>
<td>hCOL1A1</td>
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<td>ATCATGCCATAAGACAGCTGG</td>
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<td>hFN1</td>
<td>GGAGTGGATTATACCATCACTG</td>
<td>TTTCGTCTGATCTGGCCCT</td>
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</tr>
<tr>
<td>hHGF</td>
<td>CGACTGGCTTCTTTAGGCACGT</td>
<td>CTTTTCTTTTGCTCCCCTCAGC</td>
<td>197</td>
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</tbody>
</table>
### 2.2.11. Relative Quantitative RT-PCR

After RT, each sample was run in triplicate using SYBR Green JumpStart Taq ReadyMix (Sigma, S4438) according to the manufacturer’s recommendations at an annealing temperature of 60°C and 78°C for fluorescence measurement. All the primers were designed and tested so that they would not cross-react with human RNAs by running them on HepG2 cDNA. Quantitation was done by normalizing the expression levels of all the genes to the housekeeping gene Beta Actin (ActinB) using the Pfaffl method (241).

**Table 6: Primer sequences used for qRT-PCR. Abbreviations are: the preposition “r” stands for rat.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Product Size</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>rActinB</td>
<td>AAGTACTCTGTGATGGATGTTGG</td>
<td>GTGAAAGACCGAGGCTAGTAACAG</td>
<td>152</td>
<td>99%</td>
</tr>
<tr>
<td>rAlbumin</td>
<td>GATGAGAAATTGCGAAAACACTTG</td>
<td>CTTTCGGGCTTGGTTGTC</td>
<td>128</td>
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<td>rTO</td>
<td>GTCCTGGGAGGCCCATCACC</td>
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<td>TTGGCAAAACAGCTAGACA</td>
<td>115</td>
<td>99%</td>
</tr>
</tbody>
</table>

### 2.2.12. Scanning Electron Microscopy (SEM)

Flash frozen and live HUCPVCs, in 24 well plates, were fixed using Karnovsky's fixative for 10 minutes and then the samples were dehydrated in serial changes of ethanol (70%, 90%, 100% v/v), and a 9 mm of diameter disc was cut from each well using a moto-tool (Dremel, model 398). These well samples were stored in 100% ethanol until being critical point dried (Tousimis Auto Samdri 800 Critical Point Dryer), following which they were sputter coated with 10 nm of gold (Quorum SC7640 Sputter Coater) and observed using an Hitachi S-3400N Variable Pressure Field Emission SEM at 5.0 kV accelerating voltage.

### 2.2.13. Immunofluorescence

First day hepatocytes were assayed using immunofluorescence to quantify their purity by counting the whole number of nuclei and the non-albumin associated nuclei. Also 1<sup>st</sup> and 10<sup>th</sup> day cultures were also stained for albumin in order to identify the presence of
hepatocytes and the percentage of area covered by them. Immunofluorescence detection was carried after fixing the samples using zinc-formalin. The samples were later permeabilized using PBS with 0.5% triton-X for 10 min. After permeabilization, blocking followed using Tris buffer with 1% bovine serum albumin and 0.05% Tween for 1 hour. Following blocking, the samples were incubated with the primary antibody, sheep anti-rat albumin antibody (1:200, Bethyl labs, A110-134A), in blocking solution, then they were incubated with the secondary, Alexa Fluor 555 donkey anti-sheep IgG (1:600, Invitrogen, A21436), and Hoechst (1:2000) diluted in blocking solution for 1 hour. After washing the samples were photographed using an inverted fluorescent microscope (Olympus IX81 with a Photometrics CoolSnap HQ2 or Quantem 512SC camera).

The percentage of area covered by albumin positive cells (mature hepatocytes) was quantified from at least 15 fields-of-view at a 4X magnification from each biological replicate, providing an overall quantified area of at least 0.6 cm². The brightness and contrast of the images was optimized using Image Pro Analyzer 6.2.1 (Media Cybernetics), in order to increase the signal to noise ratio of the albumin signal acquired with a TRITC filter (ex545/em620). Despite the optimization, autofluorescence from dead hepatocytes could be significant; thus pictures taken in the FITC filter (ex473/em520), which would also show the autofluorescence signal but not the albumin specific signal, were subtracted from the pictures taken using the TRITC filter. Following this, the cleaned albumin signal was thresholded so that a binary image would be created and thus be quantified by dividing the number of white pixels (positive signal) by the total number of pixels of each image.

2.2.14. Statistical Analysis

Statistical analysis of isolated time points was performed by means of the Wilcoxon Rank-Sum test or Student’s T-test analysis after logarithmic transformation. Albumin secretion curves were also analysed after logarithmic transformation using the Generalized Estimating Equation (GEE), with an equicorrelated structure assumed. Each figure legend indicates which technique was used, number of samples and the p value that was considered of significance.
2.3. Results

Figure 11: Hepatocytes cocultured with HUCPVCs separated by transwell membranes or in contact in 2D and 3D configuration.
A), B) Hepatocyte albumin secretion over time. C),D) Urea in medium relative to day 1; C) day 1 was 3.6 mg/dL standard error 0.23 with 0.4 mL per well D) day 1 was 3.74 mg/dL standard error 0.5 with 0.6 mL per well. E), F) CYP activity quantified by production of Resorufin from BOMR. Grey dashed line represents the levels on day 1. A), C), E) 2D cultures. Transwell coculture = Hatched bars; Contact Coculture = Solid black bar; and monoculture = white bar. B), D), F) 3D cocultures. 3D Coculture= Black Bars; 3D Monoculture= White Bars. Error bars indicate standard error. Wilcoxon Rank-sum test used compared to monoculture. In A), differences between cultures systems analysed with GEE. 2D data represents quantification from 3 biological replicates and 3 sample replicates. 3D data represents quantification from 6 sample replicates containing a mix of 3 hepatocyte populations. *P<0.01, xP<0.05.

Our experiments were conducted to assess hepatocyte function in either contact or transwell cocultures with HUCPVCs, compared to hepatocytes alone in monoculture. Cocultures demonstrated differential improvements in the maintenance of hepatocyte function when compared to monocultures. Albumin secretion by hepatocytes in both monoculture and contact coculture decreased initially, compared to levels secreted at day 1, but both contact and transwell cocultures rescued this hepatocyte secretory capability from day 4 onwards (Figure 11A). Similar improvements in function were seen in 3D cocultures, which showed the same trend, with contact cocultures outperforming monocultures (Figure 11B). Ureagenesis decreased throughout the whole culture period in both monoculture and transwell coculture, but while it also decreased from initial values in contact coculture it stabilized from day 4 onwards (Figure 11C). Again, a similar trend was seen in 3D cocultures, although in both cocultures and monocultures a transitory increase compared to day 1 levels was observed (Figure 11D). CYP activity
was significantly increased at all time points in transwell coculture compared to either contact coculture or monoculture; showing an upward trend to day 5 and decreasing thereafter (Figure 11E). While there was also some improvement with contact cocultures, the difference between CYP activity in coculture compared to monoculture was more marked in 3D conditions (Figure 11F). The expression of hepato-specific genes, albumin, tryptophan 2,3-dioxygenase (TO) and tyrosine aminotransferase (TAT), was significantly higher in contact coculture than in either transwell coculture or monoculture (Figure 12A); while human mRNA analysis showed that HUCPVCs express several hepatotrophic factors in contact coculture (Figure 12B): the ECM proteins; decorin, fibronectin, laminin and collagen type I; and the cytokines: interleukin-6, stem cell factor, epidermal growth factor, hepatocyte growth factor and a low expression of tumour necrosis factor alpha. Connexin-43 and the Notch ligand Jagged1 were also expressed.

These functional differences were reflective of the morphology of the various cultures. Hepatocytes, identified using anti-rat albumin antibody and viewed by phase and fluorescent microscopy, showed the formation of colonies surrounded by HUCPVCs in contact cocultures (Figure 13A), and their morphology within these colonies showed the characteristic polygonal morphology of hepatocytes (Figure 13B). This was distinctly different from the hepatocytes in monoculture that showed an elongated, almost
fibroblastic, morphology (Figure 13B). Albumin expressing cells were present in all cultures although in different numbers. In monoculture they were scarce and when found, the fluorescence signal was not as strong as from cells in cocultures. We used the fluorescent emission from albumin expressing cells (albumin+) as a means to quantify the area covered by mature hepatocytes, which could be correlated with functional hepatocyte number, as there was no evidence that cell size changed with time in any of the culture geometries. This showed that contact cocultures exhibited a significant increase in the area covered by mature hepatocytes, compared to day 1 cultures (p<0.01), indicating a net proliferation of mature hepatocytes (Figure 13C); this increase was also accompanied by an increase in colony size (Figure 13A, D). Furthermore, there was an obvious difference in the area covered by albumin+ cells in contact cocultures compared to transwell cultures (Figure 13C).

Figure 13: Changes in morphology and mature hepatocyte mass during culture. A) Phase contrast pictures. A) On day 10 of culture D) on day 1 of culture. Albumin secreting hepatocytes are revealed with anti-rat albumin antibody (green). FW: 904 µm. B) Albumin immunofluorescence, albumin green and nuclei blue. FW: 218 µm. C) Quantification of area occupied by albumin+ hepatocytes relative to whole culture area on day 10. Grey dashed line indicates day 1 level. Error bars indicate standard Error. Wilcoxon Rank-sum test compared to monoculture. Data represents quantification from 3 biological replicates. *P<0.01.
2.3.1. Soluble factors effects

In transwell cocultures, HUCPVC soluble factors rescued hepatocyte albumin secretion for a transitory period but by day 10 albumin levels were at the same level as those in monoculture (Figure 11A). While there was no overall improvement in ureagenesis in transwell coculture, CYP activity was significantly higher than both monoculture and contact coculture (Figure 11E). Hepatocyte albumin gene expression on day 10 in transwell cocultures showed a small increase compared to monoculture, but was significantly higher in contact coculture (Figure 12A). At the morphological level we observed that HUCPVC soluble factors maintained a higher hepatocyte number and induced the formation of colonies that covered an increased area than those seen in monoculture, but these were significantly lower than those of either contact coculture or the initial numbers at day 1 (p<0.01) (Figure 13C). Furthermore, at day 10, not all hepatocytes were expressing albumin, suggestive of dedifferentiation (Figure 13A).

![Figure 14: Hepatocytes cultured with HUCPVC Conditioned medium (5X, 1X).](image)

Hepatocytes cultured with 5X HUCPVC CM= thick hatched bar; Hepatocytes cultured with 1X HUCPVC CM= thin hatched bar; and monoculture= white bar A) Hepatocyte albumin secretion over time. B) Urea in medium relative to day 1; day 1 was 3.7 mg/dL standard error 0.1 with 0.4 mL per well C) CYP activity quantified by production of Resorufin from BOMR. Grey dashed line represents the levels on day 1. D) Phase contrast pictures on day 10 of culture. Albumin secreting hepatocytes are revealed with anti-rat albumin antibody (green). Field Width: 904 µm. E) Quantification of area occupied by albumin+ hepatocytes relative to whole culture area on day 10. Grey dashed line indicates day 1 level. Error bars indicate standard Error. Wilcoxon Rank-sum test used compared to monoculture. In A), differences between cultures systems analysed with GEE. Data represents quantification from 3 biological replicates. *P<0.01, xP<0.05.
Since we saw some benefits from the soluble factors directly released from HUCPVCs, we explored the effects of HUCPVC conditioned medium (CM) on hepatocytes. The use of CM would offer two main advantages: the possibility of controlling the concentration of soluble factors and also the possibility of identifying the agents responsible for the effects. Thus, we tested 2 concentrations of CM, 5X and 1X. Improved albumin secretion during the initial stages of hepatocyte culture up to day 5 was shown by both 1X and 5X CM, however 5X CM had a higher effect and lasted until day 6 (Figure 14A). Although no improvements in the albumin+ cell coverage were seen at day 10, 5X CM preserved bigger colonies of cells than 1X CM or monoculture, indicating that proliferation had been induced (Figure 14D). No other benefits were seen with 5X CM or 1X CM compared to monoculture (Figure 14).

2.3.2. Non-Soluble factor effects

To examine the effect of non-soluble factors, ECM and cell-cell membrane interactions, to the exclusion of soluble factors, we used confluent flash-frozen HUCPVCs. The viability of flash frozen cells was tested using the fluorescein diacetate assay and no viable cells (fluorescent) were found on day 1 of culture nor day 15 (data not shown), indicating that the flash freezing successfully killed all the HUCPVCs, while SEM showed that the cell membranes and ECM were present (Figure 15A). Interestingly, while the albumin secretion levels of hepatocytes were not initially affected, but nevertheless decreased faster than in monoculture, this secretory function later recovered, and continued increasing for the whole culture period (Figure 15B). In addition, ureagenesis and the CYP activity remained higher than that seen in monoculture at the times measured, and the expression of albumin, TO, and TAT mRNAs on day 10 were significantly higher than monoculture (Figure 15C, D, E). In these conditions there was significantly higher albumin+ cell coverage than seen in monoculture, although still lower than that seen on day 1 (Figure 15F), the levels were similar to those seen in transwell cultures. However, the latter exhibited larger colonies but with not all cells expressing albumin, while the flash-frozen cultures had smaller colonies expressing albumin homogenously (Figure 14A, 15F).
2.3.3. HUCPVC number is proportional to the effect on hepatocytes

The number of HUCPVCs in contact with hepatocytes appears to be correlated with the maintenance of hepatocytes. Hepatocyte albumin secretion and ureagenesis were slightly
improved in ML conditions compared to monoculture, but this effect was increased in MH conditions (Figure 16A, B). CYP activity was increased significantly in MH conditions, to the level of normal HUCPVC coculture (Figure 16C). ML failed to increase CYP activity as much as the MH condition showing only an improvement on day 10. In addition, the area covered by albumin+ cells was higher in MH conditions compared to ML and both of them were higher than monoculture but did not reach day 1 levels. Only normal HUCPVCs at an initial seeding density of $1 \times 10^4$ HUCPVC/cm$^2$ were again able to facilitate mature hepatocyte expansion (Figure 16D).

From Figure 16A and B, it is possible to see that the delay on the effects that HUCPVCs have on hepatocytes is not fully associated with HUCPVC proliferation, as this delay also appears with mitomycin treated cells.

**Figure 16: Effect of HUCPVC number on hepatocytes.**

Hepatocytes cocultured with HUCPVCs at an initial seeding density of $1 \times 10^4$ cells/cm$^2$ (Normal Coculture) and Mitomycin treated HUCPVC, $2.5 \times 10^5$ cells/cm$^2$ (Coculture MH) and $1 \times 10^5$ cells/cm$^2$ (Coculture ML). Where not indicated, Coculture MH= grey hatched bar; Coculture ML= grey bar; Normal Coculture= solid black bar; and monoculture= white bar A) Hepatocyte albumin secretion over time. B) Urea in medium relative to day 1; day 1 was 4.35 mg/dL standard error 0.22 with 0.4 mL per well. C) CYP activity quantified by production of Resorufin from BOMR. Grey dashed line represents the levels on day 1. D) Quantification of area occupied by albumin+ hepatocytes relative to whole culture area on day 10. Grey dashed line indicates day 1 level. Error bars indicate standard Error. Wilcoxon Rank-sum test used compared to monoculture. In A), differences between cultures systems analysed with GEE. Data represents quantification from 3 biological replicates. *P<0.01, xP<0.05. E) Phase contrast pictures on day 10 of culture. Albumin secreting hepatocytes are revealed with anti-rat albumin antibody (green). Field Width: 904 µm.
2.4. Discussion

Our results clearly show that HUCPVCs can serve as hepatocyte stromal cells; this support involves the action of both soluble and non-soluble factors, which when independently tested show differential effects. To date, BM-MSCs have been the only MSCs that have been shown to support hepatocyte functionality and proliferation (65, 99, 107, 118, 119, 132, 144, 145, 153, 230, 242). In addition, BM-MSCs, due to their anti-inflammatory (243-245) and perhaps also their wound healing properties (97, 246), have exhibited a capacity to ameliorate liver inflammation (154, 242) and even rescue liver function (153, 154, 242, 247-251). But from a practical, therapeutic, perspective, BM-MSCs present some limitations based upon reliance on volunteer adult donors and the decreasing frequency of BM-MSCs with age (94, 95). For acute liver failure patients, rapid access to a BALS, that requires a large number of allogeneic cells, is essential. Indeed, a BALS may require 10-20 billion hepatocytes to support a patient’s failing liver (15, 17, 21), and if the hepatocyte/MSC ratio were 2:1, then 5-10 billion MSCs would also be required. An advantage of HUCPVCs as an alternative source of MSCs, in this context, is that their frequency at harvest is orders of magnitude higher than BM-MSCs; they proliferate faster (202, 203), conform with the ISCT definition of an MSC yet have similar anti-inflammatory (196) and wound healing (205) properties to BM-MSCs. Proliferative activity may be of greater importance than previously thought because we found that the HUCPVC/hepatocyte ratio is directly correlated with the strength of their effect; this is in agreement with previous reports where the fibroblast or BM-MSC/hepatocyte ratio has also shown similar trends (87, 145, 242). Interestingly, we also observed that proliferating cells had stronger effects than mitomycin growth arrested HUCPVCs. Since cell motility is not disturbed by the treatment with mitomycin (252), we hypothesize that there is either a hepatotrophic factor associated with proliferation or that mitomycin independently affects HUCPVCs so that they are not able to provide an optimal support to hepatocytes.
2.4.1. Contact Coculture Enhances Hepatocyte Function and permits net proliferation

Since hepatocytes perform a myriad of functions, it is necessary to show multi-functional improvement in order to demonstrate efficacy. Previous reports on BM-MSC-hepatocyte cocultures have shown improvement of albumin secretion, ureagenesis and CYP activity compared to monoculture (119, 145, 242). HUCPVC-hepatocyte cocultures also improved these functions, both in 2D and 3D configurations, although we also saw differences in the intensity of these effects with serum batch.

BM-MSC cocultures have also been shown to improve the maintenance of the expression of hepatocyte-specific factors (99, 145), but this has only been done in allogeneic cocultures negating the possibility of quantifying the actual expression levels. Because our cocultures were xenogeneic, we were able to design species-specific primers to quantify the expression of hepato-specific genes without any interference from the HUCPVCs employed. Thus, in contact coculture albumin, TO and TAT mRNA expression were 42, 15 and 6 fold higher respectively than in monoculture.

Additionally, using albumin+ cell fluorescence as a surrogate for mature hepatocyte mass, we were able to show that HUCPVCs were able to facilitate proliferation while restricting hepatocyte dedifferentiation in contact cocultures. Other authors have reported that BM-MSCs promote the proliferation of hepatocytes (99, 145), but to our knowledge, this has not previously been correlated with an increase in mature hepatocyte mass. Thus, for the first time, we show that a MSC is able to both increase the initial hepatocyte mass and preserve the differentiated state of the new hepatocytes.

2.4.2. Both Soluble and Non-soluble Factors contribute to hepatocyte function

Our results show that HUCPVCs express several hepatotrophic factors that have mitogenic, supportive and protective properties (65). The factors that we detected included cytokines, ECM and membrane proteins, indicating that HUCPVCs interacted with hepatocytes through both soluble and non-soluble mechanisms. Soluble factors were
able to affect albumin secretion but for a shorter time than seen in contact coculture, while non-soluble factors had a delayed effect. Furthermore, non-soluble factors improved the maintenance of ureagenesis, and hepatocyte-specific factors had a higher expression by day 10 compared to monoculture, or even cultures with HUCPVC soluble factors. Other groups have also shown that soluble factors have a positive effect on albumin secretion (114, 119). Bhandari et al (114) reported that hepatocytes cultured with 3T3-fibroblast conditioned medium had an earlier increase in albumin secretion, but the duration was shorter than that of coculture or non-soluble factors, which also had a delayed effect on albumin secretion. However, contrary to our findings the CYP activity was not affected by either soluble or non-soluble factors independently; although it is interesting to note that soluble factors were only tested by Bhandari et al using CM, and in those conditions we did not see any effect either. It is noteworthy that we observed a high increase in CYP activity levels upon hepatocyte stimulation with soluble and non-soluble-factors independently, and that 3D cocultures performed better than those in 2D in this respect. The reason why this would happen is not yet understood. It could be due to an antagonistic interaction between soluble and non-soluble factors or a contact interaction, such as gap-junction interactions, which would be impaired in our non-soluble factor model. The fact that this impairment was not manifested in 3D coculture might be due to the improvement that 3D environments provide to the hepatocyte phenotype (253).

It is important to note that despite reports on the positive effects of soluble factors (65) and the non-soluble factor Jagged1 (185) in hepatocyte proliferation, our study showed that both soluble and non-soluble stimuli are required to cause a net increase in mature hepatocyte mass. Nevertheless, we did observe an increase in the size of hepatocyte-derived colonies in conditions exposed to HUCPVC soluble factors. But, due to the lack of an evenly distributed albumin expression, at day 10, these cultures failed to show any increase in the mass of mature hepatocytes. On the other hand, hepatocytes cultured with flash frozen HUCPVCs showed small colonies with strong homogeneous albumin expression. These two observations suggest that in our conditions, soluble factors had a strong mitogenic effect while non-soluble factors preferably preserved the mature hepatocyte phenotype.
2.5. Conclusion

In conclusion, HUCPVC provide hepatocytes with required signals for the extended maintenance of their mature phenotype in vitro. Soluble and non-soluble factors play differential roles in these interactions.
3. CHAPTER 3: The HUCPVC-hepatocyte coculture: effects on hepatocyte structure and functional polarity; and the HUCPVC phenotype

* The author of this thesis performed all the work presented within this chapter.
Abstract

Hepatocyte culture is a useful tool for the study of their biology, and the development of bioartificial liver systems. However, many challenges have to be overcome since hepatocytes rapidly lose their normal phenotype in vitro. Approaches such as the use of extracellular matrix (ECM) proteins, cytokines and coculture with other cells have improved the maintenance of hepatocyte functions and morphology. We have recently demonstrated that Human Umbilical Cord Perivascular Cells (HUCPVC) are able to provide support to hepatocytes; as judged by improved secretory and metabolic activity as well as the expression of selected hepato-specific genes. In the present study we go further into exploring the effects that HUCPVCs have in the functional polarization, and both the internal and external organization, of hepatocytes. Also, we investigate HUCPVC-hepatocyte cross-talk by tracking both the effects of HUCPVCs on hepatocyte transcription factors and those of hepatocytes on the expression of hepatotrophic factors in HUCPVCs. Our results show that HUCPVCs maintain both the storage of glycogen and the functional polarity of hepatocytes, ex vivo, as judged by the secretion of fluorescein into bile canaliculi (BC), for at least 40 days. Transmission electron microscopy (TEM) revealed that hepatocytes in coculture organize in an organoid-like structure embedded in ECM surrounded by HUCPVCs. In coculture hepatocytes displayed a higher expression of C/EBPα, implicated in maintenance of the mature hepatocyte phenotype; and HUCPVCs upregulated hepatocyte growth factor and Jagged1 indicating that these genes may play important roles in HUCPVC-hepatocyte interactions. Conclusion: HUCPVCs support the maintenance of the hepatocyte ultrastructure and functional polarity; while cross-talk with hepatocytes upregulates hepatotrophic factors, particularly HGF, IL6 and JAG1 in HUCPVCs.
3.1. Introduction

Bioartificial liver systems have been in development, for 30 years, using both cells and synthetic components (18). Since hepatocytes are the main functional cell of the liver, their ex-vivo culture is of key importance for the effective development of bioartificial liver systems (20). Of known cells that can provide liver supplementation, primary hepatocytes predominate, since the current human liver cell lines, which include the C3A line, immortalized adult and foetal hepatocytes, have a significantly lower metabolic capacity (21, 23). However, primary hepatocytes do not maintain their normal polarity and functions adequately ex vivo (104, 254) but, while they do, they provide adequate metabolic activity for bioartificial liver systems, for up to 89 hours in the HepatAssist (21). Thus the study of hepatocyte biology, and the improvement of both their maintenance and expansion ex vivo are important therapeutic goals. Usually in standard culture conditions, hepatocytes show a significant decrease in viability within the first 48 hours, and continue to dedifferentiate thereafter. After the second day of culture they start showing changes in their original morphology, becoming less polygonal, flattened, often apolar, also more translucent and less granular (106, 211, 254). These changes become more apparent with time in culture.

In order to improve hepatocyte maintenance, different approaches have been used including the use of supplements and hormones (212, 215, 216), cytokines (115), ECM proteins (106, 220) and coculture of hepatocytes with a variety of other cells types (87). Previously it has been shown that hepatocytes embedded in collagen type I (COL-I) repolarize forming BC for up to 10 days while maintaining an improved functionality (104, 255, 256). Furthermore, the coculture of hepatocytes with bone marrow stromal cells (BM-MSC) has shown the presence of functional BC up to 21 days and it appears that COL-I is of importance in the interaction of the two cell types (144). The formation of BC permit elimination of xenobiotics and other waste products (104, 257). This may aid hepatocyte maintenance by diminishing toxic effects on hepatocytes. In addition, the BM-MSC-hepatocyte coculture has shown an improved maintenance of hepatocyte-related factors and C/EBPα (99), which is a regulator of hepatocyte differentiation (258).
We have recently shown that HUCPVCs, a mesenchymal cell type, have hepatocyte supportive capacity (Chapter 2). HUCPVCs are cells that are acquired from the Wharton’s Jelly surrounding the umbilical cord vessels. They have shown many similarities with BM-MSCs (196, 202, 204) while exhibiting a higher proliferation rate (202) and colony forming unit-fibroblast frequency at harvest compared to human BM-MSCs (203). We showed that hepatocytes, in coculture with HUCPVCs, increased their number and improved the maintenance of hepatocyte functions. These included albumin secretion, ureagenesis and a higher expression of the hepato-specific factors, albumin, tryptophan 2,3-dioxygenase and tyrosine aminotransferase (Chapter 2).

We show herein that HUCPVCs are able to maintain the functional polarity of hepatocytes, and therefore functional BC, for even a longer time (40 days) than has been previously shown in the literature for cocultures with BM-MSCs (21 days (144)) or any other cell type. The maintenance of polarity is also shown to be correlated with the high expression of the transcription factor C/EBPα, glycogen storage and the formation of desmosomal junctions between hepatocytes. Finally, we also demonstrate that HUCPVCs adapt to the presence of hepatocytes by upregulating the expression of hepatotrophic factors.

3.2. Material and Methods

3.2.1. Animals

Male Wistar Rats weighing 200–300 g (Charles River Laboratories, Montreal, Canada) were used for hepatocyte isolation. The animals were cared in accordance with the guidelines set by the Animal Research Ethics of the University of Toronto and the National Institutes of Health.
3.2.2. Hepatocyte Isolation and culture

Hepatocytes were isolated by two-step collagenase perfusion of the liver as previously described by Moldeus et al. 1978 (239). The cells were further purified by Percoll centrifugation (240). The hepatocytes acquired were plated at a concentration of 28.3x10³ hepatocytes/cm² in M1 conditions: COL-I (25 µg/cm², BD, Franklin Lakes, NJ) gel coated plates with Williams E medium (Sigma, Canada) supplemented with 2% FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin (Invitrogen, Canada), 50 ng/ml Gentamicin (Sigma), 10 ng/ml EGF (R&D systems, Minneapolis, MN), 1mM ascorbic acid 2-P, 20 mM HEPES, 0.1 U/ml Insulin, 10⁻⁷ M Dexamathasone and 10mM Nicotinamide (Sigma); from day 4 of culture 1% DMSO (Sigma) was also added to the culture medium.

3.2.3. HUCPVC culture and source

HUCPVCs were provided by Tissue Regeneration Therapeutic Inc, Toronto, Canada. The cells were expanded in α-MEM (Invitrogen) supplemented with 10% FBS and antibiotics: penicillin G 167 U/mL, amphotericin B 0.3 mg/mL, gentamicin 50 mg/mL (Sigma). Medium was changed 2-3 times a week, and cells were passaged at 80% confluence. For coculture experiments, HUCPVCs were pooled from at least 5 different donors.

3.2.4. Coculture

The day following the seeding of hepatocytes, after media samples were collected, HUCPVCs were laid on top of hepatocytes at a concentration of 10x10³ cells/cm². Media samples were collected daily for albumin measurements and stored at -20°C. M1 conditions, described above, were used for both cocultures and monocultures.

3.2.5. Immunofluorescence

Immunofluorescence detection was undertaken after fixing the samples using zinc-formalin. Sheep anti-rat albumin antibody (1:500, Bethyl laboratories, Montgomery, TX)
and/or rabbit anti-COL-I (1:40, Millipore, Billerica, MA) were used as primary antibodies; and Alexa Fluor 555 donkey anti-sheep IgG and/or anti-rabbit (1:600, Invitrogen) were used as secondaries. Nuclei were also stained with Hoechst (1:2000).

The percentage area covered by albumin positive cells (mature hepatocytes) was quantified as previously described (Chapter 2).

### 3.2.6. BC functionality

The functionality of BC was identified using the method described by Barth and Schwarz (259). This method detects BC functionality by their capacity to secrete fluorescein produced intracellularly from uptaken fluorescein diacetate (259), a secretion mediated by the multidrug resistance-associated protein 2 (255). Briefly, hepatocytes were incubated at 37°C for 20 minutes with fluorescein diacetate (Sigma) at a concentration of 10 µg/ml in culture medium. The cultures were then washed 4 times with PBS and then medium was replaced for viewing under the fluorescent microscope.

### 3.2.7. TEM

After removing the medium, the culture samples were washed once with PBS and then fixed using Karnovsky's fixative for at least 2 hours. The fixative was replaced with 0.1 M sodium cacodylate buffer (pH 7.3) and the samples were stored at 4°C until processing. Subsequently, the samples were post-fixed for an hour with 1% OsO4 and then rinsed once with 0.1 M sodium cacodylate buffer with 0.2 M sucrose (pH 7.3). After dehydration with serial changes of ethanol, samples were embedded in Quetol-Spurr resin. Ultrathin sections were stained with 2% uranyl acetate for 15 minutes and 0.2% lead citrate for 5 minutes. The sections were viewed and imaged on a Tecnai 20 TEM (FEI, Hillsboro, OR).
3.2.8. Periodic Acid Schiff staining

Briefly, after fixing the cultures, they were washed 3 times with distilled water and then incubated in periodic acid solution for 7 minutes, then washed 3 times and incubated in Schiff’s reagent for 18 minutes (reagents from Sigma). After washing the samples 6 times, pictures were taken using light microscopy.

3.2.9. Hepatocyte Functional Assays:

3.2.9.1. Albumin ELISA

Method previously described in Chapter 2.

3.2.9.2. Urea secretion and cytochrome P450 (CYP) activity measurements

At some time points both cocultures and monocultures were incubated at 37°C with 5% CO₂ for 1 hour while being rocked (200 µl in 24 well plates) in Williams E medium supplemented with antibiotics, 1 mM ammonium chloride (stock 400 µM) and 5 µM Vivid BOMR substrate (stock 2mM in acetonitrile, Invitrogen). Following incubation, 100 µl samples were transferred to 96 well plates and fluorescence (excitation 530, emission 605) was read at 37°C to detect CYP activity. Urea was quantified using QuantiChrom Urea Assay Kit (Bioassay systems, Hayward, CA).

3.2.10. RNA Isolation, Reverse Transcription and Relative Quantitative RT-PCR (qRT-PCR)

For the measurement of hepatocyte mRNA expression, hepatocyte cocultures and monocultures were cultured in M1 conditions. To measure the changes in hepatotrophic factor expression in HUCPVCs, HUCPVCs were cultured alone (control) and with hepatocytes (treatment) in two conditions: M1; and M2, a condition previously used for coculture (Chapter 2): cells were cultured directly into plastic dishes in DMEM (Sigma)
supplemented with 10% FBS, 1 µM Dexamethasone, 1x ITS (MP Biomedicals, Solon, OH) and 100 U/ml Penicillin, 100 µg/ml Streptomycin.

Total RNA was isolated using Tri Reagent (Sigma), then genomic DNA elimination and reverse transcription was performed using QuantiTect Reverse Transcription Kit (Qiagen, Canada) followed by the company’s recommended procedures.

Each sample was run in triplicate using SYBR Green JumpStart Taq ReadyMix (Sigma) according to manufacturer’s recommendations at an annealing temperature of 60°C and 78°C for fluorescence measurement. All rat primers pairs (Supplementary Table 1) were designed and tested so that they would not cross-react with human RNAs. Human primer pairs (Supplementary Table 2) were tested on hepatocytes and none of them had any reaction except for FN1; however, the reaction was negligible relative to FN1 expression in naive HUCPVCs. Quantitation was done by normalizing the levels of all the genes to the housekeeping gene’s using the Pfaffl method (241): for rat, Beta Actin (rActinB) and for human, Beta-2-microglobulin (hB2M).

3.2.11. Statistical Analysis

Statistical analysis of isolated time points was performed by means of the Wilcoxon Rank-Sum test or Student’s T-test analysis after logarithmic transformation. Each figure legend indicates which technique was used, number of samples and the p value that was considered of significance.
3.3. Results

3.3.1. Hepatocytes in coculture have functional and morphological polarity

Our experiments were conducted to assess hepatocyte functional polarity in coculture with HUCPVCs, compared to hepatocytes in monoculture. Cocultures demonstrated improvements in the maintenance of hepatocyte functional polarity and overall function, when compared to monocultures. In coculture, functional BC, detected by their secretion of fluorescein, were found extensively distributed on days 16 (Figure 17A-C), 20, 30 and 40 (Supplementary Figure 1). No BC were detected in monocultures. At the microstructural level the BC, detected in cocultures, contained microvilli and the surrounding hepatocytes formed tight junctions between each other followed sometimes by desmosomes (Figure 17D).

Figure 17: Cocultures present functional BC and mitochondria differ from monoculture.
A) Phase contrast picture of hepatocytes in coculture; B) same field as A showing well defined BC by their secretion of fluorescein. Cells treated with fluorescence diacetate and observed under the fluorescence microscope after 20 min. C) A and B merged. Bile secretion is identified within the gaps between hepatocytes seen under phase contrast microscopy (yellow arrows). A)-C) 20 day. Field Width=514 μm. D) In coculture, hepatocytes contain peroxisomes (P), form BC, tight junctions (black arrow head) and desmosomes (white arrow head) can be found. Day 16. Bar 500 nm. E) Hepatocyte mitochondria. Day 16. Bar 250 nm. RER and SER, Rough and Smooth endoplasmic reticulum, respectively; M, Mitochondria; Hep, Hepatocyte; HUC, HUCPVC.
3.3.2. Ultrastructural and Morphological difference between monoculture and coculture

We investigated the effects of such polarization in the ultrastructure and organization of the cells. Internally, the mitochondria of hepatocytes in coculture had a higher electron density and cristae compared to the ones in monoculture (Figure 17E).

At an organizational level, hepatocytes in coculture organize in 3D organoid-like structures containing BC and surrounded by ECM and HUCPVCs (Figure 18A). Cocultures with GFP+ HUCPVCs reveal that HUCPVCs are present in most areas where hepatocytes are found (Supplementary Figure 2). Monocultures, on the other hand, have flat hepatocytes that form a monolayer with no further complexity and lack BC (Figure 18B).

![Figure 18: Organizational culture structure and ECM distribution.](image)

A) Hepatocytes in coculture form organoid-like structures coated by HUCPVCs and ECM. B) Hepatocytes in monoculture are flattened and form a monolayer. A), B) Bar 2.5 µm. C) ECM containing collagen and other components is found between HUCPVCs and hepatocytes. Bar 500 nm. Hep, Hepatocyte; HUC, HUCPVC. D) Light microscope picture of hepatocytes in coculture (hepatocytes identified by their high contrast) E) COL-I covers most of the culture. Immunofluorescence showing the presence of COL-I in the ECM, same field as D. F) Most hepatocyte colonies are covered by COL-I leaving empty spaces only where no hepatocytes are present. E merged with Hoechst nuclear staining. Hepatocytes colonies can be identified in sites of high concentration of nuclear staining. D)-F) Field Width: 2228 µm.
The ECM between hepatocytes and HUCPVCs contains fibrillar collagen (Figure 18C). The presence of COL-I as well as fibronectin was confirmed using immunofluorescence; showing COL-I coating on most of the hepatocyte structures (Figure 18D-F) and the presence of fibronectin within the ECM (Supplementary Figure 3B). COL-I and fibronectin were identified within both hepatocytes and HUCPVCs (Supplementary Figure 3A-D). In monoculture, no COL-I fibres were identified. Some external non-fibrillar COL-I was however found on some hepatocytes and non-parenchymal cells (Supplementary Figure 4).

3.3.3. HUCPVCs support the maintenance of hepatocyte functionality

To confirm that polarization was accompanied by an improved maintenance of the mature phenotype, we analysed the secretion of albumin, ureagenesis, CYP activity, glycogen storage and expression of hepato-related genes. Cocultures showed an increase in albumin secretion over time (Figure 19A), reaching a peak around day 18. The maintenance of higher levels compared to monoculture was however seen up to day 40 (data not shown). Also, while ureagenesis was initially lower in cocultures compared to monoculture, the levels were higher in the longer term (≥16 days) (Figure 19B). Cocultures had an improved CYP activity on days 8 and 12 compared to monoculture. The levels remained similar to day 1 afterwards (p>0.1). Monoculture, on the other hand, had a temporary negligible CYP activity, which increased from day 16, having a higher activity on day 20 compared to coculture (Figure 19C). On day 20, cocultured hepatocytes also showed strong staining for glycogen while monocultures had almost no, or very low, staining (Figure 19D); these differences were also seen on days 30 and 40 (data not shown). At the mRNA level, an overall higher expression of hepato-related genes was seen in cocultures compared to monoculture on day 20 (Figure 19E).
3.3.4. Effective proliferation of hepatocytes in coculture

After confirming that the polarized hepatocytes had an improved maintenance of the overall mature phenotype, we investigated whether these characteristics would also allow them to have any effective hepatocyte mass increase during the course of culture. We used the fluorescent emission from albumin expressing cells (albumin+) as a surrogate to quantify the area covered by mature hepatocytes, which could be correlated with
functional hepatocyte number, as there was no evidence that cell size changed with time in any of the culture geometries. This showed that cocultures exhibited a significant increase in the area covered by mature hepatocytes, compared to day 1 cultures (p<0.01), indicating a net proliferation of mature hepatocytes (Figure 20A). On the other hand, monocultures exhibited a significant decrease in mature hepatocytes. Nevertheless, an extensive increase in non-albumin+ hepatocytes was often seen (Figure 20D); while in other cases, cells in monoculture died off and left empty spaces (not shown). Noteworthy is the fact that hepatocytes in both coculture and monoculture eventually condense, an organization not found at day 1 (Figure 20B-D).

Figure 20: Effect of HUCPVCs in hepatocyte expansion.
A) Hepatocytes proliferate and maintain their phenotype in coculture. Quantification of area occupied by albumin+ hepatocytes relative to whole culture area. Data represents quantification from 3 biological replicates. *P<0.01; Wilcoxon Rank-sum test. Error bars indicate standard Error. B) First day hepatocytes are mostly spread; C) hepatocytes in coculture aggregate (day 20) and; D) hepatocytes in monoculture proliferate while losing their phenotype (day 20). B-D): Phase contrast pictures of cultures. Albumin secreting hepatocytes are revealed with anti-rat albumin antibody (green). Field Width: 904 µm.

3.3.5. Effects of hepatocytes on HUCPVCs

In vivo, liver stromal cells and hepatocytes interact with each other significantly affecting their phenotypes (74, 87). Because of this, we also explored the possible effects that hepatocytes could have on the expression of hepatotrophic factors produced by HUCPVCs. We initially tested for the mRNAs previously shown to be expressed in cocultured HUCPVCs (Chapter 2): the ECM proteins: decorin, fibronectin, laminin and COL-I; the cytokines: interleukin-6 (IL6), stem cell factor, epidermal growth factor, HGF and tumour necrosis factor alpha; the membrane proteins: Connexin-43 and the Notch ligand JAG1. We were not able to accurately quantify tumour necrosis factor alpha,
COL-I or epidermal growth factor, since their expression was too low to allow a precise quantification. In the conditions used for the above cocultures (M1), we did not find any significant changes in the expression of any of the ECM proteins tested (Supplementary Figure 5A). However the expression of cytokines was different: HGF showed a significant upregulation (Figure 21A); stem cell factor showed an upregulation tendency (p=0.06) (Supplementary Figure 5A); IL6 had a very low expression in this condition, which did not allow us for an accurate quantification. JAG1 was also found to be upregulated in HUCPVCs (Figure 21A).

After testing upregulation in M1 conditions, we decided to also explore the changes in M2 conditions. We retested mRNAs that were found to be significantly different; ECM genes, as cells were seeded directly into plastic, likely changing ECM expression; and IL6, since it has been shown to mediate MSC-hepatocyte interactions (119). We found, again, no significant changes in ECM gene expression (Supplementary Figure 5B). In addition, HGF was upregulated, although less than in M1 conditions (Figure 21B). However JAG1 was only minimally increased (p>0.1)(Supplementary Figure 5B). HUCPVCs also showed a significant IL6 upregulation, of more than 16 fold, in coculture (Figure 21B).

HUCPVCs express JAG1 at the protein level (Supplementary Figure 6) and may increase the expression of JAG1 mRNA upon
coculture with hepatocytes. TEM demonstrated that HUCPVC-hepatocyte contact does occur in coculture (Figure 21C).

3.4. Discussion

3.4.1. Hepatocytes remain polarized in coculture

In the healthy liver, hepatocytes are polarized; they present basolateral and canalicular domains (255, 260, 261). The canalicular domains are used for the secretion of bile and the basolateral domains have contact with blood, being employed for the uptake, secretion and excretion of substances (260, 261). In vitro, functional and morphological polarity can be identified by the presence of secretory BC (257, 262). This functionality has been previously shown for up to 21 days in other cultures and cocultures (144, 255, 257, 259). Thus, the extension of this functional outcome until day 40 in our HUCPVC-hepatocyte cocultures represents a significant increase. Interestingly, the first signs of polarization in the liver bud only appear upon interaction with the mesoderm (261), thus it is possible that HUCPVCs, being mesenchymal cells, provide cues similar to the ones provided by the mesoderm to induce polarization.

3.4.2. Coculture facilitates the maintenance of the Hepatocyte phenotype

We have previously reported that hepatocyte maintenance was significantly improved in the presence of HUCPVCs (Chapter 2). In the present work, in an aim to further improve hepatocyte maintenance, we changed the culture conditions (M2) to one of higher complexity (M1) where other factors that have been shown to improve hepatocyte maintenance such as nicotinamide, epidermal growth factor, ascorbic acid, DMSO and COL-I were added (115, 212, 215, 216, 263). In M1 conditions, both cocultures and monocultures were improved; HUCPVCs still showed functional stromal capacity by improved albumin secretion, glycogen storage, hepato-related gene expression and
facilitated a net increase in mature hepatocyte mass. The higher expression of C/EBPα gives further evidence of a more differentiated state, as this transcription factor is involved with the activation of numerous hepato-related genes (258). A temporal improvement of CYP activity was seen in coculture, although hepatocytes in monoculture also showed increased CYP activity from day 16. This paradoxical increase could be due to the possibility that at a certain stage of dedifferentiation, cells reach a state similar to foetal hepatocytes, leading to a temporal increase in CYP activity (23, 264, 265).

Ultrastructural studies also revealed that hepatocyte mitochondria differed between monoculture and coculture, indicating that hepatocytes had different cell phenotypes (266, 267). Interestingly, the mitochondria of cocultured hepatocytes appeared to have a closer similarity to those found in vivo, as reported by Bahr (268) (Figure 17E). We also observed that mitochondria of cocultured hepatocytes had increased numbers of cristae compared to those in monoculture which would correlate with an increased enzymatic activity (269).

At an organizational level, we found, similarly to other reports, that hepatocytes in monoculture presented a flat morphology (106, 211, 254), whereas in coculture a more complex structure was formed. Hepatocytes were surrounded by ECM and enveloped by HUCPVCs, similar to the organization reported for BM-MSC-hepatocyte cocultures (144). Previously, we (Chapter 2) showed that non-soluble factors were able to exert positive effects on the maintenance of the mature hepatocyte phenotype; our ultrastructural observations indicate that HUCPVCs may interact with hepatocytes through both ECM and contact interactions.

### 3.4.3. ECM and hepatocytes

In agreement with other reports (145, 225), the internal expression of COL-I was detected in hepatocytes in both coculture and monoculture. However, despite its external presence in hepatocytes and the few non-parenchymal cells in monoculture, no COL-I fibres were observed. Additionally, while HUCPVCs expressed COL-I, fibres were preferentially formed on or around hepatocytes. This indicated that the environment created by the
HUCPVC-hepatocyte coculture was required for the deposition of COL-I into the ECM, in agreement with Goulet et al’s (225) observations, where both hepatocytes and liver endothelial cells had internal expression of COL-I, but fibres were only formed in coculture. Other cocultures, including BM-MSC’s, have shown similar COL-I and other ECM protein distribution (144, 226, 270) and Gu et al (145) showed that COL-I expression was increased within both hepatocytes and BM-MSCs in coculture. The deposition of other ECM proteins has shown to be modulated by the interaction of hepatocytes and stellate cells (270); from our results it appears that COL-I deposition is also regulated by the HUCPVC-hepatocyte interaction.

3.4.4. Upregulation of hepatotrophic factors in coculture

Cell interactions are usually two-way; cross-talk occurs between cells in coculture (74, 87, 99, 145). Herein we assayed the effect of hepatocytes on HUCPVCs, focusing on the changes of expression of hepatotrophic factors. Our rationale was that a MSC exposed to an inflammatory environment upregulates factors associated with immunomodulation (271), thus hepatotrophic factors would potentially get upregulated upon exposure to hepatocytes. We used M1 and M2 conditions to prove this hypothesis. In both conditions upregulation of HGF occurred. HGF is mitogeneic, induces maturation and has a cytoprotective effect on hepatocytes (65). IL6 which has been shown to play an important role in the BM-MSC-hepatocyte interaction (119, 230) did show increase in M2; but its expression could not be quantified in M1, which was supplemented with nicotinamide, an inhibitor of IL6 expression (272).

HUCPVCs express the Notch ligand JAG1 (Supplementary Figure 3E, F), which upon coculture in both M1 and M2 conditions tends to be upregulated. This upregulation is of interest since it has also been seen in BM-MSC-hepatocyte cocultures (99). It is possible that JAG1, as previously suggested (99), is involved in hepatocyte proliferation in the MSC-hepatocyte interaction, as JAG1-Notch signalling, on its own, has mitogeneic characteristics (185). However, in a previous report we showed that contact interactions do not cause a net increase in hepatocyte mass (Chapter 2). Thus, it is possible that other effects can be taking effect. In fact, it has recently been shown that Notch signalling can
have hepato-protective effects (273), thus indicating that other roles of the JAG1-Notch interaction still require to be explored.

3.5. Conclusion

In conclusion, HUCPVCs support the maintenance of the hepatocyte ultrastructure, functional polarity and maintain a higher expression of C/EBPα; while they are also influenced by hepatocytes, upregulating the levels of at least two hepatotrophic factors.
3.6. Supplementary Material

Supplementary Table 1: Primer sequences used for qRT-PCR. Abbreviations are: the preposition “r” stands for rat.

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<td>GTGTAAACCGAGCTCTAGAAG</td>
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<td>rAlbumin</td>
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<td>rTO</td>
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<td>rTAT</td>
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<td>TGGCCAACACAGCTAGACA</td>
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<td>rHNF1α</td>
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<td>rC/EBPα</td>
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Supplementary Table 2: Primer sequences used for qRT-PCR. Abbreviations are: the preposition “h” stands for human.

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<td>CGGTATCTCTGAAGGTTCCC</td>
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<td>96%</td>
</tr>
<tr>
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<tr>
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<tr>
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<td>hJAG1</td>
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Supplementary Figure 1: BC found in cocultures on day 40.
A) Phase contrast picture of hepatocytes in coculture; B) same field as A showing well defined BC by their secretion of fluorescein. Cells treated with fluorescence diacetate and observed under the fluorescence microscope after 20 min for bile secretion. C) A and B merged. Field Width: 904 µm.
**Supplementary Figure 2: Hepatocyte-HUCPVC coculture.**
Left, merged image of rat albumin immunofluorescence and Hoechst nuclear stain. Middle, GFP-transduced HUCPVCs. Right, Merged image rat albumin immunofluorescence (orange) and GFP signal from HUCPVCs. FW: 2228 µm

**Supplementary Figure 3: COL-I and FN1 expression.**
A),C) Hepatocytes identified by their albumin expression within coculture. Nuclei blue (Hoechst) D) Expression of external and internal fibronectin on same field as A. (1:200 Rabbit fibronectin antibody, Sigma) B) Expression of extracellular and internal collagen in same field as C. FW: 218 µm.

**Supplementary Figure 4: External COL-I.**
A) Light microscope picture of hepatocytes in monoculture. E) Immunofluorescence showing the ECM presence of collagen type I in same field as D. F) E merged with Hoechst (blue) nuclear staining. Field Width 2228 µm.
Supplementary Figure 5: Change in expression of hepatotrophic factors in HUCPVCs after coculture with hepatocytes.
A) Cocultured for 20 days in M1. B) Cocultured for 10 days in M2. Error bars indicate standard deviation from 3 samples. Each coculture contains hepatocytes from a different subject. T-test after logarithmic transformation. *P<0.05.

Supplementary Figure 6: JAG1 expression in HUCPVCs.
Nuclei (blue, Hoechst) and expression of JAG1 (green, 1:50, Rabbit anti- Jagged1, Abcam). Field Width: A) 904 µm and B) 150 µm respectively.
4. CHAPTER 4: Liver Therapy: Two different Mesenchymal Stromal Cells for different therapeutic needs

* The author of this thesis performed most of the work within this chapter, however Yimin Wang cultured and processed the samples for microarray; Corey Ng and Joseph Ng worked on the acquisition of the IDO transcript and activity levels.
Abstract

The inflammatory response upon liver injury plays an important role in the onset of liver fibrosis and further hepatocyte injury, which may ultimately lead to liver failure. The attenuation of inflammation and the rescue of hepatocytes is therefore of utmost importance to allow any recovery. Mesenchymal stromal cells (MSC) from bone marrow (BM) have shown promise as rescuers, and preventers, of liver failure. We have previously described an alternative, more plentiful, source of MSCs coming from the umbilical cord perivascular tissue, Human Umbilical Cord Perivascular Cells (HUCPVC). Here we present data showing that although HUCPVCs and BM-MSCs share many similarities, they also exhibit functional differences that can provide advantages for liver therapeutic needs. Both MSCs support hepatocytes but HUCPVCs tend to have an earlier effect in ureagenesis while BM-MSCs have a strong effect in the cytochrome P450 activity of hepatocytes. Also, while HUCPVCs have a higher expression of PD1-ligands, there is higher inducibility of indoleamine 2,3-dioxygenase (IDO) in BM-MSCs. Thus MSCs from different sources can serve as hepatocyte stromal cells and mitigate inflammation, but they present differences in the manner they affect hepatocytes and the expression of hepatotrophic and anti-inflammatory factors.
4.1. Introduction

To date, the only successful way to treat liver failure is transplantation; however such approach is only available to a selected patient group. While Acute Liver Failure (ALF) has a very low incidence, its mortality rate is 60-90% (7); and Acute On Chronic Liver Failure (AOCLF), which is much more common, accounted for approximately 1.1% of deaths in Canada between 2003-2007 (6). Both ALF and cirrhosis, the main cause of AOCLF, involve inflammation and cellular damage (1, 247). In normal conditions upon liver injury, an inflammatory response is triggered to allow regeneration (1, 274). This requires the precise orchestration of hepatocyte and non-parenchymal cell proliferation, apoptosis suppression, angiogenesis, extracellular matrix remodelling and immunomodulation (47, 274-276). However, in some cases, inflammation does not abate, further injuring the organ and, depending on the intensity, leading to either ALF or chronic injury, in which the continuing regenerative response eventually loses equilibrium leading to fibrogenesis, which may later develop into cirrhosis (1, 274). Therefore, diminishing inflammation and ameliorating recovery of the liver cell content are promising alternatives to transplantation.

Mesenchymal stromal cells (MSC) have been shown to secrete trophic factors that aid healing (97, 246); moreover they have both anti-inflammatory and hepatotrophic capacity. MSCs have been already used to treat inflammatory complications (243) and are able to at least inhibit both T and B cell proliferation, through different mediators such as the PD-1 pathway and IDO (97, 196, 277, 278). MSCs have also rescued hepatocytes in vitro, improving their survival, restoring their polarity and functionality, and even inducing their proliferation (65)(Chapter 2). The ways by which MSCs affect hepatocytes include cytokines, extracellular matrix and even contact interactions (65). Thus, MSCs may be excellent candidates to supply the therapeutic needs of ailing livers.

Indeed, MSCs have shown much potential to treat liver disease. Murine BM-MSCs have rescued injured livers with acute inflammation, preventing the onset of fibrosis (247); also, non-hematopoietic cells from the bone marrow (BM), which contain MSCs, ameliorate CCl4 induced liver fibrosis in mice (248). In rats, infused BM-MSCs have a
significant effect in reversing liver fibrosis induced by $\text{CCL}_4$ and dimethylnitrosamine (250). Furthermore, in a model of acute liver failure in rats, BM-MSC secreted factors diminished liver inflammation, improved survival, increased liver regeneration and improved liver function (242). Kharaziha (251) showed that 8 cirrhotic patients that were treated with autologous BM-MSCs had an improvement of the disease with no adverse effects. In addition, Human Wharton’s jelly MSCs were shown by Tsai et al (279) to protect and heal the rat liver from fibrosis caused by $\text{CCL}_4$ while diminishing liver inflammation.

As discussed, most research has shown the potential of adult BM derived MSCs for liver therapy, although neonatal MSCs have also shown a similar capacity (196, 279)(Chapter 2). In our laboratory, MSCs were found in the Wharton’s Jelly surrounding the umbilical cord vessels, we call these cells Human Umbilical Cord Perivascular Cells (HUCPVC). HUCPVCs have many similarities with BM-MSCs, including an immunosuppressive phenotype, while exhibiting a higher proliferation rate and colony forming unit-fibroblast frequency at harvest compared to BM-MSCs (196, 202, 204). Interestingly, Wharton’s jelly MSCs have recently been shown to exhibit greater immunosuppression than BM-MSCs (280).

Thus we wished to directly compare BM-MSCs and HUCPVCs in the light of their potential for liver therapeutics. Thus, in the present study we first highlight their similarities and focus on their capacity as putative hepatocyte stromal cells; and then compare their anti-inflammatory profile. Our results show the potential strength of each cell type for different liver therapeutic needs.
4.2. Materials and Methods

4.2.1. Animals

Male Wistar Rats weighing 200–300 g (Charles River Laboratories, Montreal, Canada) were used for hepatocyte isolation. The animals were cared for in accordance with the guidelines set by the Animal Research Ethics of the University of Toronto.

4.2.2. Hepatocyte Isolation and culture

Hepatocytes were isolated by two-step collagenase perfusion of the liver as described previously (Chapter 2). The hepatocytes acquired were plated at a concentration of 28.3x10^3 hepatocytes/cm^2, and maintained in hepatocyte culture medium (HCM): DMEM (Sigma, Canada) supplemented with 10% FBS, 1 µM Dexamethasone (Sigma), 1x ITS (MP Biomedicals, Solon, OH) and 100 U/ml Penicillin, 100 µg/ml Streptomycin (Invitrogen, Canada).

4.2.3. HUCPVC culture and source

HUCPVCs were provided by Tissue Regeneration Therapeutics Inc, Toronto, Canada. The cells were expanded in α-MEM (Invitrogen) supplemented with 10% FBS and antibiotics: penicillin G 167 U/mL, amphotericin B 0.3 mg/mL, gentamicin 50 mg/mL (Sigma). Medium was changed 2-3 times a week, and cells were passaged at 80% confluence. For coculture experiments, HUCPVCs were pooled from at least 5 different donors.

4.2.4. Rat Non-parenchymal (NP) Liver cells Isolation and culture

After liver perfusion, hepatocytes were left to settle for ten minutes. The supernatant resulting from this settling was taken, centrifuged at 300 x g for 5 min and the pelleted
cells were seeded using DMEM (Sigma) supplemented with 10% FBS and 100 u/ml Penicillin, 100 µg/ml Streptomycin (Invitrogen). While some hepatocytes were among the population, after 3 passages (P3) only non-parenchymal cells remained as judged by morphology. Thus post-P3 cells were used for experiments. For coculture experiments, a NP cells were pooled from 3 different rats.

4.2.5. Human BM-MSC culture and source

BM-MSCs were also provided by Tissue Regeneration Therapeutics Inc. They were cultured in the same way as described for HUCPVCs. For coculture experiments, BM-MSCs were pooled from at least 5 different donors.

4.2.6. Hepatocyte Coculture

The day following the seeding of hepatocytes, media samples were collected and then, HUCPVCs, BM-MSCs or NP cells were laid on top of hepatocytes at a concentration of 10x10^3 cells/cm². Following this, the medium was changed daily, and samples were collected for albumin measurements and stored at -20°C. The medium used for both cocultures and monocultures was HCM, described above.

4.2.7. Microarray

HUCPVCs and BM-MSCs were cultured on 6 well plates using the conditions previously described. Upon reaching around 80% confluency the RNA was isolated using Tri Reagent (Sigma) and later purified using RNeasy MinElute cleanup kit (Qiagen, Canada) as per manufacturer’s instructions. RNA purity and yield was determined using the NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE), and quality with Agilent 2100 bioanalyzer (Agilent Technologies, Canada). 8 HUCPVC biological replicates and 7 BM-MSC biological replicates were used for microarray analysis using the GeneChip Human Gene 1.0 ST array (Affymetrix, Santa Clara, CA) as per manufacturer’s instructions.
4.2.8. Microarray Analysis

The raw data was imported into GeneSpring GX11 software (Agilent Technologies) and it was normalized using Robust Multichip Average (RMA) normalization with the addition of value 16 to expression values (RMA16). Signals below the 20th percentile of raw signal were filtered out to minimize background as per manufacturer’s recommendation. Entity significance was tested using t-test with an $\alpha$ of 0.005 and Benjamin-Hochberg corrections.

The data acquired was compared with public data from other tissues and cell type transcriptomes through hierarchical clustering using the Euclidean distance metric and centroid linkage rule. The cell types and tissues used for comparison were: adipose tissue derived MSC (GSE18662), amnion derived MSC (GSE28385), BM-MSC profiles from another research group (GSE20631), osteoblasts (GSE11414), monocytes (GSE15791), CRL-2522 a dermal fibroblast cell line, ES cells, hepatocytes, melanocytes (GSE23034), primary dermal fibroblasts (GSE24621), vascular smooth muscle cells (GSE30004), HUVEC (GSE12011), whole bone marrow (RIKEN Research Center for Allergy and Immunology), adipose tissue (GSE20571), liver, heart and muscle (Affymetrix).

4.2.9. Immunofluorescence

Immunofluorescence detection was carried after fixing the samples using zinc-formalin. Sheep anti-rat albumin antibody (1:500, Bethyl laboratories, Montgomery, TX) used as primary antibodies; and Alexa Fluor 555 donkey anti-sheep IgG (1:600, Invitrogen) was used as secondary. Nuclei was also stained with Hoechst (1:2000).

The percentage of area covered by albumin positive cells (mature hepatocytes) was quantified as previously described (Chapter 2).
4.2.10. **Hepatocyte Functional Assays:**

4.2.10.1. **Albumin ELISA**

Method previously described in Chapter 2.

4.2.10.2. **Urea secretion and cytochrome P450 (CYP) activity measurements**

Method previously described in Chapter 2.

4.2.11. **IDO induction by Interferon-γ (IFN-γ)**

HUCPVCs and BM-MSCs were cultured in α-MEM (Invitrogen) supplemented with 10% FBS and 100 U/ml Penicillin, 100 µg/ml Streptomycin (Invitrogen). Two groups were set for analysis of the effect of IFN-γ in the upregulation of IDO gene expression and activity: group 1 was the control group with no IFN-γ supplementation and group 2 was stimulated with 500 U/ml of recombinant human IFN-γ (2 x 10^7 IU/mg, US biological, Swampscott). All groups were seeded at the same cell concentration and stimulation started 4.5 hours after seeding and lasted for 70 hours. IDO expression and activity were then determined.

4.2.12. **Determination of IDO activity**

To measure IDO activity, cells were harvested and the number of living cells was determined by trypan blue exclusion. The cells of each sample were resuspended at a concentration of 4000 or 8000 cells per µl of PBS containing a protease inhibitor cocktail (BioShop, Canada) and lysed by 4 cycles of freezing and thawing. After centrifugation (5000 x g for 10 minutes), the supernatants were used as cell extracts; 45 µl of cell extract was mixed with 45 µl of IDO reaction buffer (100 mM PBS, 40mM ascorbic-acid-phosphate, 20µM methylene blue, 200 µg/ml catalase, 800µM L-Tryptophan, pH 6.5). After incubating for 30 min at 37°C, 18 µl of 30% trichloroacetic acid were added to stop
the reaction and another incubation at 50°C for 30 minutes followed (hydrolysis of N-formylkynurenine to L-kynurenine). Samples were then spun down (5000 x g for 10 minutes) and 100 µl of the supernatant were mixed with 100 µl of Ehrlich reagent on a 96 well plate and allowed to react for at least 10 minutes at room temperature. The absorbance of the samples was then read with a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, VT) at 490 nm. The absorbance values were converted to kynurenine moles using an L-kynurenine standard (0-5000 µM). The protein concentration, used for normalizing the kynurenine values, was determined using the Bio-Rad Protein Assay Kit II (Biorad, Canada) as per manufacturer’s instructions.

4.2.13. RNA Isolation, Reverse Transcription and Relative Quantitative RT-PCR (qRT-PCR)

Total RNA was isolated using Tri Reagent (Sigma) then genomic DNA elimination and reverse transcription was performed using the QuantiTect Reverse Transcription Kit (Qiagen, Canada) followed by the company’s recommended procedures. Each sample was run in triplicate using SYBR Green JumpStart Taq ReadyMix (Sigma) according to manufacturer’s recommendations at an annealing temperature of 60°C and 78°C for fluorescence measurement. All rat primer pairs (Supplementary Table 1) were designed and tested so that they would not cross-react with human RNAs by running them on HepG2 cDNA. Human primer pairs (Supplementary Table 3) were tested on hepatocytes and none of them had any reaction except for fibronectin (FN1); which reacted but the levels found were approximately 40 fold less than the levels presented by pure HUCPVCs. Quantitation was done as previously described (Chapter 2)

4.2.14. Telomerase Activity

HUCPVC telomerase activity was detected using the TRAPEze® XL Telomerase Detection Kit (Millipore, Canada) as per manufacture’s instructions.
4.2.15. Statistical Analysis

Statistical analysis of isolated time points was performed by means of the Wilcoxon Rank-Sum test or Student’s T-test analysis after logarithmic transformation. Each figure legend indicates which technique was used, number of samples and the p value that was considered of significance.

4.3. Results

4.3.1. HUCPVC and BM-MSC share a similar phenotype

HUCPVCs, as previously mentioned, have shown many properties that have indicated that they belong to the MSC type. To further this knowledge, we did a direct comparison of the transcriptome of HUCPVCs with that of BM-MSCs, and other cell types and tissues from public data. Using hierarchical clustering of all the samples, the validity of the clustering was confirmed by observing known related samples being clustered, i.e. hepatocytes clustered with liver tissue, and the lack of aggregation of cells from different origins. Thus, the clustering showed that both HUCPVCs and BM-MSCs belong to a group which only contains spindle shaped mesenchymal cells; that included BM-MSCs and other MSCs acquired and cultured by other groups (Figure 22A). Hierarchical clustering based on the expression profile of putative MSC hepatotrophic factors (65) showed similar aggregation to the whole transcriptome analysis, but in this case a subgroup was formed with most MSCs, excluding amnion membrane derived MSCs, and including vascular smooth muscle cells. A high expression of Interleukin-6 (IL6) and an overall higher expression of the putative hepatotrophic profile compared to the other cells was characteristic of this subgroup (Figure 22B).
Figure 22: Similarities between HUCPVCs and BM-MSCs.

A) Hierarchical clustering of different cell types and tissues according to transcriptome. B) Hierarchical clustering of different cell types according to the expression of putative hepatotrophic factors. High (red), medium (black) and low (green) levels. AMNION-MSC, AT-MSC, BM-MSC (1,2,3), HUCPVC, mesenchymal stromal cells from different sources. FIBROBLASP, primary dermal fibroblasts; FIBROBLAST, dermal fibroblast cell line; V-SMC, vascular smooth muscle cell; AT, adipose tissue; ES, embryonic stem cells; BM-HSC, bone marrow derived hematopoietic stem cells. C) Telomerase activity on HUCPVCs as measured using TRAPEze. HeLa protein extracts were used as Positive Control. D) Number of MHCII molecules in HUCPVCs either expressed Higher, Similar or Lower than BM-MSCs at the mRNA level; Genes are considered different when they differ more than 1.5 times between cell types and are significantly different at an \( \alpha \) of 0.005. A, B and D: Data from Affymetrix Human ST 1.0 Microarray.

Focusing on HUCPVCs and BM-MSCs, we found similarities in factors that are relevant for cell therapy. One of them was telomerase expression: HUCPVCs had similar levels of the telomerase reverse transcriptase mRNA to BM-MSCs (\( p > 0.1 \)). This is also confirmed by the fact that HUCPVCs lacked telomerase activity (Figure 22C), as we have previously reported (197).

Also, it is known that both MSCs are non-alloreactive and lack the expression of MHCII molecules (196, 203); thus we decided to investigate in more detail the relative expression of MHCII and the costimulatory molecules CD80 and CD86. We considered only differences that were greater than 1.5 fold and with a \( p \)-value (\( p \)) below 0.005. We found that out of the 14 MHCII molecules analysed, 12 were similar between the two cell types and 3 were lower in HUCPVCs (Figure 22D). The expression of costimulatory molecules was similar; HUCPVCs having a 0.7 (\( p = 0.002 \)) and 0.87 (\( p = 0.015 \)) fold expression of CD80 and CD86, respectively, relative to BM-MSCs. This was also
corroborated in our lab group at the protein level using flow cytometry on both cell types (208).

Furthermore, when the expression of factors that could serve liver healing was compared, it was found that most were similarly expressed in basal conditions: Out of 12 hepatotrophic factors analysed, 9 were similar; of 7 antiapoptotic factors, only 1 was different; all the angiogenic and antifibrotic cytokines were similarly expressed; 13 out of 16 anti-inflammatory factors showed similarity (Supplementary Table 4); and out of 22 chemokines analysed 15 did not show significant difference (Supplementary Figure 7). Despite the many similarities found, 3 chemokines were expressed at a significantly lower level in HUCPVCs while 4, all of which are pro-angiogenic (281), were significantly higher than BM-MSCs (Supplementary Figure 7).

4.3.2. Differentially expressed hepatotrophic factors

In basal conditions 3 hepatotrophic genes were expressed at significantly lower levels in HUCPVCs than BM-MSCs: the cytokine stem cell factor (KITGL), the proteoglycan decorin (DCN) and the notch ligand jagged 1 (JAG1). Hepatocyte Growth Factor (HGF) also showed a lower expression but it was significant only at a p<0.01 (Supplementary Table 4).

Due to the differences seen in basal conditions we decided to explore whether they were maintained upon coculture with hepatocytes due to cell-cell crosstalk. Using qRT-PCR we found that the HUCPVC/BM-MSC gene expression ratio was significantly changed. While we were unable to acquire confident quantification of collagen type I (COL-I), epidermal growth factor (EGF) and tumour necrosis factor α (TNFα) due to their low expression relative to the housekeeping gene, most of the other genes showed variation relative to basal conditions: DCN became similarly expressed between the two cell types; while laminin (LAMB1), HGF, IL6 and Connexin-43 (GJA1) showed a higher expression in HUCPVCs (fold difference>2.5 and p<0.05) (Figure 23). Thus, HUCPVCs
had a higher expression of 4 hepatotrophic factors compared to 2 in BM-MSCs (KITLG and JAG1) in coculture conditions, a significantly different profile to the one observed in basal conditions.

Figure 23: mRNA expression of selected hepatotrophic factors, detected by qRT-PCR, in HUCPVCs compared to human BM-MSCs after being in coculture with hepatocytes. Normalized to BM-MSCs and B2M. T-Test after logarithmic transformation. Error bars indicate standard deviation from 3 cocultures, each containing hepatocytes from a different subject. *P<0.05.

4.3.3. HUCPVCs support hepatocytes differently from BM-MSCs

Due to the fact that the two cell types have a different hepatotrophic profile, both in basal conditions and in coculture with hepatocytes; and that they can support hepatocytes in vitro; we directly compared their effect on hepatocytes in addition to that caused by rat liver NP cells.

Using the fluorescent emission from labeled albumin expressing cells (albumin+) as a means to quantify the area covered by mature hepatocytes, which could be correlated with functional hepatocyte number, we found that mature hepatocytes in coculture with either HUCPVCs or BM-MSCs were covering a similar area at the end of culture (p>0.1). Also, the area covered by mature hepatocytes in MSC cocultures was higher compared to monoculture and the NP-hepatocyte coculture (p<0.01)(Figure 24A). NP-hepatocyte cocultures had a higher area covered by mature hepatocytes than in monoculture (p<0.01), although it was significantly lower than on day 1 (Figure 24A). On the other hand, both MSCs had an increase in the covered area by mature hepatocytes (p<0.05) (Figure 24A) and the hepatocyte colony size (Figure 24B).
Figure 24: Effect of HUCPVCs, BM-MSCs and NPs on mature hepatocyte mass.
HUCPVC (HUcocul), BM-MSC (BMcocul) and NP (NPcocul) -hepatocyte cocultures. A) Quantification of area occupied by albumin+ hepatocytes relative to whole culture area. Wilcoxon Rank-Sum test. Error bars indicate standard error from 3 biological replicates. *P<0.01 compared to monoculture B) Phase contrast pictures of cultures at day 10. Albumin secreting hepatocytes are revealed with anti-rat albumin antibody (green). Field Width: 904 µm.

MSC-hepatocyte cocultures also showed a higher effect at the functional level. While monocultures continuously decreased the medium albumin levels, an increase was seen in cocultures after an initial decrease period. Noteworthy was the fact that MSC-hepatocyte cocultures increased albumin secretion much more than NP-hepatocyte cocultures. However, HUCPVC cocultures, by the last day of culture, were outperformed by BM-MSC’s as the albumin levels suddenly decreased (Figure 25A). Despite that, both MSC-hepatocyte cocultures showed improved (p<0.01) expression of hepato-specific proteins: albumin and tryptophan 2,3-dioxygenase (TO). Tyrosine aminotransferase (TAT) was however only upregulated significantly (p<0.05) in HUCPVC cocultures (Figure 25B).

The maintenance of ureagenesis was improved in HUCPVC cocultures compared to monoculture on the last two measurements performed; while BM-MSC’s showed significance in the last measurement only and NP cell’s failed to show any improvement (p<0.01) (Figure 25C). On the other hand, only BM-MSC cocultures showed an increase in the CYP activity (Figure 25D).

Since some reports have shown that MSCs have the capacity to transdifferentiate into hepatocytes (65); thus interfering with our measurement of ureagenesis and CYP activity, we assayed for differentiation in the cocultures. Using RT-PCR we looked for human
albumin mRNA in cocultures. We found none in either HUCPVC or BM-MSC cocultures (data not shown).

Figure 25: Effect of HUCPVCs, BM-MSCs or NPs on hepatocyte function and phenotype. Where not indicated hepatocytes cocultured with HUCPVCs, black bars; Hepatocytes cocultured with BM-MSCs, striped bar; hepatocytes cocultured with NPs, hatched bar; and monoculture, white bar A) Secretion of albumin by hepatocytes over time B) Relative albumin, TO and TAT gene expression. Normalized to ActinB and the expression on the first day. Error Bars indicate standard deviation from 3 biological replicates. T-test after logarithmic transformation. C) Urea in medium relative to day 1 where it was 3.7 mg/dL standard error 0.1 with 0.4 mL per well D) CYP activity quantified by production of Resorufin from BOMR®. Grey dashed line, level on day 1. A), C) and D) Wilcoxon Rank-sum test. Error bars indicate standard error from 3 biological and 2 sample replicates. *P<0.01 compared to monoculture.

4.3.4. Differences in the expression of Anti-inflammatory factors

Because inflammation plays an important role in the onset and continuation of liver disease, it is highly relevant to also characterise the differences in expression of anti-inflammatory factors. From the microarray data we identified 3 factors that were highly expressed by HUCPVCs compared to BM-MSCs. Two of these factors were the PD1-ligands and the other was Interleukin-11 (IL11). We decided to reconfirm the difference of the PD1-ligands due to the fact that their expression has been directly linked to MSC immunosuppression (277, 282). qRT-PCR confirmed that PD-L1 expression was higher in HUCPVCs compared to BM-MSCs, that had almost undetectable expression (Figure
The expression of PD-L2 was about 2 times higher in HUCPVCs (Figure 26A). However, since PD-L2 may be expressed at the mRNA level but not always at the protein, and is rarely found in non-lymphoid organs (283), we also confirmed its presence in HUCPVCs using immunofluorescence (Supplementary Figure 8).

Figure 26: PD-1 ligands and IFN-\(\gamma\) induced IDO expression in HUCPVCs and BM-MSCs.

IDO secretion is another factor that has been shown to be used by BM-MSCs to inhibit T cell proliferation (278, 284). The microarray revealed that both BM-MSCs and HUCPVCs had a similar expression of it in basal conditions; thus indicating that the expression was negligible, as BM-MSCs only express it upon simulation with IFN-\(\gamma\) (278, 284). While lack of expression and activity were indeed seen in unstimulated cells; IFN-\(\gamma\) stimulation significantly upregulated IDO mRNA, and IDO activity, in both cell types. However, BM-MSCs showed a higher upregulation of the activity compared to HUCPVCs, while they had a similar upward tendency at the mRNA level (Figure 26B, C).
4.4. Discussion

MSCs have shown considerable potential in the healing of numerous types of injuries and illnesses (65, 97, 197, 204, 246). In addition, their negligible immunogenicity has been the focus of much attention (97, 197, 246). The use of MSCs for both extending the function of hepatocytes in bioartificial liver systems and for liver treatments per se is feasible since they have been shown to serve as putative hepatocyte stroma, as anti-inflammatory agents (97, 196, 278), and can provide other trophic properties that facilitate healing (246).

4.4.1. HUCPVCs can serve for cell therapeutics

In previous reports it has been shown that HUCPVCs and BM-MSCs possess similar characteristics regarding their MSC phenotype including their lack of telomerase (196, 197, 202-204). However, their transcriptomes had not been previously compared. We found that HUCPVCs and BM-MSCs cultured in the same conditions shared many similarities, although changing culture conditions did introduce differences (Figure 22A). This parallels the results of Wagner et al (285) who showed that BM-MSC morphology changed with the culture conditions employed. Similarly, the constitutive chemokine expression in both cell types was generally similar, suggesting that they are able to attract immune cells in order to exert their anti-inflammatory effects (286).

Interestingly, of the 7 chemokines differentially expressed, the 4 upregulated in HUCPVCs were angiogenic (281), although angiogenic cytokines were not significantly different (Supplementary Table 4). Hypoxia plays an important role in cirrhosis and other liver diseases and, despite neovascularization, the new vessels fail to resolve the hypoxia due to their immaturity (287, 288). Due to the role of perivascular populations in the maturation of vessels (288, 289), it is possible that HUCPVC-induced angiogenesis may be accompanied by HUCPVC-facilitated maturation. Indeed, the high expression of FLT1—29 fold that of BM-MSCs— and FLK1 — 3 fold — suggests that
HUCPVCs can react and sequester vascular endothelial growth factor more effectively and thus play an important role in the modulation of vessel formation (290, 291).

### 4.4.2. Differential effects on hepatocytes

In the liver, NP and parenchymal cells (hepatocytes) require a balanced interaction (74, 87); this interaction is however lost in vitro and their reunion is not able to completely restore the original homeostasis (63, 292). However, numerous reports have shown that the coculture of all or part of the NP cell population with hepatocytes helps to maintain a more differentiated state in the latter (63, 87, 292). Herein, for the first time, we show that both HUCPVCs and BM-MSCs are able to provide a higher degree of support to hepatocytes than their native stromal cells. The reason why NP cells fail to provide strong support might be related to the fact that the main cell types, stellate and sinusoidal endothelial cells, suffer from a change in phenotype in vitro (74). MSCs, too, change phenotype (285) but it appears that their tendency to restore balance is greater and thus they provide more adequate support to hepatocytes, an important property for therapeutics.

The support provided by HUCPVCs and BM-MSCs can be attributed to their hepatotrophic phenotype, levels of which differ between the two cell types (Figure 23, Supplementary Table 4). This in part explains why these two cell types show differences in their effects on both ureagenesis and CYP activity. These differences appear to suggest that due to the accelerated maintenance of ureagenesis by HUCPVCs, the latter might be preferable for systems that aim to eliminate hyperammonaemia, that can cause critical complications in liver failure (5). On the other hand, the improvement of CYP activity in the presence of BM-MSCs indicates that they could be more appropriate for treating cases of xenobiotic related-liver injury and for creating more stable testing devices for pharmacology.

Nevertheless, despite the benefits that MSCs can provide to hepatocyte maintenance, it can be argued that the improved hepatotrophicity might not be exclusive of MSCs. Indeed it has been shown that other cells can provide support to hepatocytes (87, 242).
Yagi et al (242), directly compared BM-MSCs and NIH 3T3-J2 fibroblasts, a highly hepatotrophic cell line (228), and their results indicated that both cells had similar supportive capabilities in normal conditions. However, BM-MSCs showed improved hepato-protection and support of ureagenesis compared to fibroblasts in the presence of serum from ALF subjects. Furthermore, BM-MSCs had a marked capacity to rescue rats from ALF; in part by reducing the acute inflammation (242).

4.4.3. Different level of factors involved in the PD1 and IDO Pathways

Our results showed that both MSCs had a similar anti-inflammatory profile (Supplementary Table 4). However 3 interesting differences were identified. IL11 had a higher expression in HUCPVCs; IL11 has been regarded as an anti-inflammatory cytokine, having the capacity to reduce the secretion of inflammatory cytokines and reduce colitis (293). Still, despite being expressed in BM-MSCs in basal conditions, it has not yet been shown to be directly implicated in BM-MSC immunosuppression (294). On the other hand, the other differences observed were related to a higher expression in HUCPVCs of the two PD1 ligands, which have shown to play important roles in MSC immunosuppression (277, 282). Specifically, Augello et al (277) showed that the two ligands were involved in the inhibition of T and B cell proliferation by BM-MSCs, and they suggested the predominance of redundant, cytokine based, mechanisms in interactions lacking contact. However, it is still possible that the PD1 pathway has effects without the need of cell contact as it has been found that at least PD-L1 can also be released as a soluble protein (295). Interestingly, PD-L1 has been recently found to be a modulator of angiogenesis, suggesting again that HUCPVCs may be better angiogenic modulators compared to BM-MSCs (295).

While we found no other anti-inflammatory factors to be differentially expressed in basal conditions. IDO, which is not constitutively expressed, was upregulated to higher levels in BM-MSCs compared to HUCPVCs upon IFN-γ stimulation. This corroborates the finding of Prasanna et al (280) comparing Wharton’s jelly MSCs and BM-MSCs. IDO is another factor that has been shown to play an important role in MSC immunosuppression
and it appears to work independently of the PD1 pathway, having an additive effect in the inhibition of T cell proliferation (282). However, IDO has shown to have a cooperative effect on the establishment of B cell mediated inflammatory responses (296).

Interestingly, both the PD1 and IDO pathways appear to be involved in the modulation of liver inflammation (276, 297, 298), thus indicating a putative mechanism for MSC mediation of liver inflammation. The fact that HUCPVCs and BM-MSCs have different levels of expression of the factors involved in the two pathways, suggests that they could also have differential effects in acute and chronic inflammation. Still, to date we only know that the two cell types can similarly regulate peripheral blood lymphocyte proliferation (196), an effect that can be modulated by both pathways. It is possible that a comparative analysis of the effects of the two MSC types in specific immune cell populations, i.e. T and B cells, NK, macrophages, could shed some light on the importance of the differential expression of IDO and PD1 ligands. Similarly, experiments where these two MSCs are directly compared in the way in which they affect ALF and chronic hepatitis are required to elucidate the possible differences at the functional level.

4.5. Conclusion

In conclusion, both mesenchymal cell types are able to support hepatocytes and mitigate inflammation. But, their effects can vary due to the differences in their hepatotrophic and anti-inflammatory profiles. HUCPVCs and BM-MSCs have different effects on hepatocytes: HUCPVCs have a faster effect on the maintenance of ureagenesis while BM-MSCs increase CYP activity. This difference appears to be due to differences in the levels of expression of hepatotrophic factors. In addition, the expression of anti-inflammatory factors differs between the two cell types. Specifically HUCPVCs have a stronger expression of PD-1 ligands and BM-MSCs secrete higher amounts of IDO. Thus each cell type can address different therapeutic needs.
4.6. Supplementary Material

Note: Supplementary Table 1 can be found within the Supplementary Material of the previous chapter.

Supplementary Table 3: Primer sequences used for qRT-PCR. Abbreviations are: the preposition “h” stands for human.

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<th>Reverse</th>
<th>Product Size</th>
<th>Efficiency</th>
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Supplementary Table 4: Putative hepatotrophic, and wound healing factors that can be secreted by BM-MSCs being evaluated in HUCPVCs in relation with BM-MSCs. Affymetrix Human ST 1.0 Microarray.

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<td></td>
<td></td>
<td>TGFb (246, 293)</td>
<td>0.9</td>
<td>0.2133</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Cell Surface/Soluble</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>HLA-G (246)</td>
<td>0.8</td>
<td>0.0284</td>
<td>/</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>Cell Surface</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PD-L1 (277)</td>
<td>3.4</td>
<td>0.0001</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PD-L2 (277)</td>
<td>1.7</td>
<td>0.0001</td>
<td>++</td>
</tr>
</tbody>
</table>
Supplementary Figure 7: HUCPVC chemokine expression relative to BM-MSCs.
Left, Number of Chemokines in HUCPVCs either expressed Higher, Similar or Lower than BM-MSCs at the mRNA level. Right, Chemokines differentially expressed between HUCPVCs and BM-MSCs in basal conditions. Data from Affymetrix Human ST 1.0 Microarray.

Supplementary Figure 8: Expression of PD-L2 in HUCPVCs.
Nuclei (blue, Hoechst) and expression of PD-L2 (orange, murine monoclonal anti-PD-L2- 1:125, R&D systems MAB1224.). Left picture, FW:1200 um and Right, FW: 300 um.
5. CHAPTER 5: General Discussion, conclusions and future work
The hypothesis underlying the work undertaken herein was that HUCPVCs are putative stromal cells for hepatocytes and are able to support them through soluble and non-soluble factors, improving their functionality, polarity, morphology and net proliferation. The work presented through the previous chapters demonstrates that HUCPVCs are able to provide hepatocytes with required cues for an improved maintenance of their mature phenotype. The ways and outcomes of the HUCPVC-hepatocyte interaction negate the possibility of disproving the hypothesis in the various conditions used during this project. This finding brings new opportunities to the field of hepatology.

In the following general discussion, we will address additional issues not covered in the previous chapters: the rationale for the methods used; the advantages and limitations of HUCPVCs as putative hepatocyte stromal cells; the influence of soluble and non-soluble factors on hepatocyte functionality; additional analysis on the fact that HUCPVC trophic characteristics differ from BM-MSC’s; conclusions and future perspectives.

5.1. Rationale for the methods used

In order to test whether HUCPVCs were able to provide support to hepatocytes we decided to use a xenogeneic system. While hepatocytes from different species have different levels of functionality (23), the xenogeneic system has the important advantage of allowing the independent study of the two cell populations without the need for their sorting or even their removal from their acquired tissue architecture. Furthermore, since the studies in male rat hepatocytes have shown much correlation with later studies in human hepatocytes (105, 221), the use of rat hepatocytes would provide enough evidence for later human tests.

To elucidate the effect of HUCPVCs we chose five general approaches that would allow us to have an overall understanding of the effects over a wide range. Albumin secretion was chosen due to its ubiquitous use on testing hepatocyte functionality (65, 87). Using ELISA, we were able to specifically measure the secreted amounts of albumin over time, without any interference from the bovine albumin in the foetal bovine serum or the
possible human albumin from transdifferentiated cells. While albumin expression is an indication of a mature hepatocyte phenotype, it does not necessarily correlate with an improvement of other functions. Thus we also measured the amount of urea synthesized; ureagenesis in mammals serves as the main way for the elimination of ammonia, a toxic metabolite from aminoacid and protein catabolism (301) which can cause encephalopathy, one of the major complications in liver failure (3-5). Another important metabolic function of hepatocytes is the processing of xenobiotics and hormones; to catabolize these components the body uses the cytochrome P450 (CYP) enzymes. Of all the different isoforms, we chose a method that would determine the activity of the hepatic CYP3A as it is the most abundant CYP in the liver being implicated in metabolizing more than 50% of the drugs currently used (302). However, the substrate used in our method may also interact with CYP1A family members (Manufacturer’s information).

Additionally we investigated the levels of expression of rat transcripts that are associated with the mature hepatocyte phenotype: albumin, tryptophan 2,3-dioxygenase (TO) and tyrosine aminotransferase (TAT) were tested in all the previous chapters, while C/EBPα and HNF1α were only tested in chapter 2. Testing for the levels of expression of the albumin transcript allowed us to corroborate the results acquired at the protein level while being able to address the proportional increase independent of any proliferation. In Appendix 3, it is shown that the level of albumin transcript does correlate with the rate of its secretion into the medium, indicating that mRNA levels were a good indicator of the functional levels. The expression of TO and TAT, which are enzymes in charge of metabolizing the aminoacids tryptophan and tyrosine respectively, rapidly decrease during hepatocyte dedifferentiation, thus they were an indication of the level of aminoacid metabolism in hepatocytes and thus their function. HNF1α and C/EBPα are transcription factors that influence many hepatocyte functions (258, 303-305)(Table 7); for instance, both factors have been shown to act synergistically to activate the expression of albumin (306).

Finally we used albumin immunofluorescence to quantify the purity of harvests (>98%), better identify cell morphology, and identify differentiated hepatocyte distribution and determine the ratio of covered area. The ratio of albumin+ covered area was used as a surrogate to discover if there was an increase or decrease in the functional hepatocyte
mass. This approach allowed us to determine if there was effective proliferation. Effective proliferation required two events, an increase in hepatocyte number without dedifferentiation. The use of nucleoside analogues to thymidine, such as bromodeoxyuridine or 5-ethynyl-2'-deoxyuridine, combined with albumin immunofluorescence would only show the generation of new cells while not necessarily indicating an increase in cell mass, as other cells could have died in the process. Metabolic assays for quantification are not specific for cell types, thus it would not directly target hepatocytes. Furthermore, as hepatocytes can be in different states of differentiation, their metabolism would be different, directly affecting the results. Counting methods such as the combination of trypan blue exclusion with albumin immunofluorescence requires the disruption of the formed tissue and thus can be accompanied by considerable loss in cell viability due to the fact that hepatocytes formed highly confluent colonies. Thus, our method of quantifying the area covered by albumin+ cells in relation with the whole culture area provided a simple approach that, while not perfect, produced reproducible results between experiments and samples. An important flaw is that the increase in hepatocyte mass can be underestimated in coculture, as it was found, through TEM, that hepatocytes tended to aggregate in coculture, and in some sections of the colony they could form a bilayer. However due to this flaw, the assay is conservative in indicating any increase in mature hepatocyte mass. Thus the finding that cocultures generated an increase in mature hepatocyte mass, indicates that this increase is in fact significant.

Table 7: Gene expression regulated by HNF1α and C/EBPα.

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>Known targeted genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNF1α</td>
<td>Albumin, glucose-6-phosphatase, alpha fetoprotein, CRP, phosphoenolpyruvate carboxykinase, α-1-antitrypsin, aldolase Beta, α and β fibrinogen, apolipoproteins, CYP2E1, insulin-like growth factor binding protein-1, liver fatty acid binding protein, phenylalanine hydroxylase and clotting factors (303, 304)</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>Albumin, glucose-6-phosphatase, alpha fetoprotein, TAT, α-1-acid glycoprotein, bilirubin UDP-glucuronosyltransferase, serum amyloid A, glycoegen synthase, ureagensis, haptoglobin, CYP2B, complement C3, Factor IX (258, 305)</td>
</tr>
</tbody>
</table>
5.2. Advantages and limitations of HUCPVCs as putative hepatocyte stromal cells

Through all the previous techniques we demonstrated that HUCPVCs enhance the overall state of hepatocytes compared to standard monoculture. Additionally in Chapter 3, we demonstrated the long term maintenance of the function of bile canaliculi and the improved storage of glycogen in cocultures; on the contrary, in monoculture canaliculi were not preserved and glycogen storage was low or absent. Additionally, in pilot studies, where only hepatocytes from one subject were used, we were also able observe that HUCPVC-hepatocyte cocultures were better than the collagen sandwich culture configuration as shown in Appendix 4. This culture configuration has been shown to be one of the most effective monocultures, providing a highly improved environment for hepatocytes compared to monoculture on a single layer of collagen; there is improvement in the preservation of the original morphology, albumin secretion, ureagenesis and CYP activity (105, 106, 221). HUCPVC-hepatocyte cocultures were compared to collagen sandwich monocultures by means of their albumin secretion at different culture densities, 3 different densities of hepatocytes (High, 118.4x10^3 cells/cm^2, Medium, 39.5 x10^3 cells/cm^2, Low, 13.2x10^3 cell/cm^2) with 3 different densities of HUCPVCs (High, 27x10^3 cells/cm^2, Medium, 9x10^3 cells/cm^2, Low, 3x10^3/cm^2). We found that hepatocytes at high densities waived the beneficial effects of HUCPVCs (p>0.1 using General Estimating Equation-GEE). This was possibly due to the quiescence and lower metabolic rates of hepatocytes at high density (174, 307) or a diminished stromal effect resulting from diminished cell-cell interactions; the HUCPVC-hepatocyte cocultures with medium (p<0.05) and low hepatocyte densities (p<0.001) showed an improved prolonged maintenance of albumin secretion compared to the sandwich monoculture. Interestingly in this setting, where HUCPVCs were proliferating, unlike in Chapter 2, we saw no significant effect of the HUCPVC culture density (data not shown), as exposed by a lack of difference even with an α of 0.1 using GEE analysis. This can be explained by the fact that while in Chapter 2 we showed that there was a correlation between the number of mitomycin treated HUCPVCs and the effect they had on hepatocytes, we also showed
that proliferation played an important role, more so in the increase in albumin secretion. Thus, since albumin secretion was the evaluator in the pilot experiments, the differential effect caused by the density of HUCPVCs in culture was hidden by the improvement conferred by unknown factor(s) associated with proliferation.

In Chapter 2 and Chapter 3, we showed the effects of HUCPVCs in hepatocytes in different culture conditions, as we were searching to improve the culture conditions used in Chapter 2 and also prove that HUCPVCs were capable of being hepatotrophic despite the environment to which they were exposed. Additionally a problem that was noticed in Chapter 2 regarding the fast proliferation of HUCPVCs was overcome with the conditions used in Chapter 3; as some of the initial cultures barely made it till day 10 of culture, while in the new conditions cultures were able to continue at least for 40 days. Furthermore, since the new conditions had a lower concentration of serum, 2% instead of 10%, the change in serum lot had no significant effect on the secretion of albumin as observed when testing 3 different lots (data not shown). However despite the improvements, hepatocytes in both conditions had a considerable decrease in ureagenesis, which in coculture was slowed down but did not recover to initial levels. This observation indicates that while HUCPVCs do provide many of the required cues to hepatocytes for the maintenance of their mature phenotype, still some factors are missing. To be able to address the possible missing factors we ought to go back to the original hepatocyte in the liver. In such an environment, hepatocytes primarily interact with each other, the ECM in the space of Disse, SCs and SECs. In the HUCPVC-hepatocyte cocultures, hepatocytes interact with each other and HUCPVCs, which being mesenchymal and perivascular cells assume the role of SCs in vivo. Additionally the establishment of an ECM in coculture can be creating a similar environment to that one created by the space of Disse. However an important component, the SECs, is still missing and could be the reason why hepatocytes do not completely recover their in vivo phenotype. There are two ways to address the question on whether a third endothelial component would actually significantly improve the state of the cocultures. One way would be the coculture of HUCPVCs, hepatocytes and SECs together. While it is known that SECs as SCs, in vitro, dedifferentiate, losing their fenestrations and the capacity to produce homeostatic factors, it has also been shown that coculture of SECs with
hepatocytes or SCs improve the maintenance of the original phenotype (86, 308).
Furthermore, the use of mesenchymal-hepatocyte cocultures appears to extend the effect of the maintenance of the SEC phenotype (308). Thus, a coculture of HUCPVCs, hepatocytes and SECs could potentially create a system where stability is reached by a simultaneous maintenance of SECs and hepatocytes through the interaction of all the cell types within the triculture. However for engineering purposes, the use of primary SECs is very limited, comprising up to only 12% of the liver cells. Thus the second approach that we propose, a more viable alternative for practical applications, could be using Human Umbilical Cord Endothelial Cells (HUVECs) instead of SECs. While HUVECs lack the phenotypic characteristics of SECs, they are endothelium-derived and are as readily available as HUCPVCs. Furthermore, HUVECs can be acquired from the same cord and have already shown to be able to improve hepatocyte function at least through soluble factors (309).

5.3. The influence Soluble and Non-Soluble factors on hepatocyte functionality

In Chapter 2, we provide evidence of the contribution of both HUCPVC soluble and non-soluble factors to the HUCPVC-hepatocyte interaction. However it was seen that HUCPVCs in contact coculture were not able to provide an enhancement of CYP activity of a greater or at least similar order than in transwell or flash-frozen HUCPVCs coculture. In contact coculture of HUCPVCs and hepatocytes, both soluble and non-soluble factors are interacting with hepatocytes and thus it would be expected that the improvement of CYP activity would be at least similar or greater than when those two factors are evaluated separately. However, in the test using transwell cocultures or culture with flash frozen HUCPVCs, there were some limitations, or missing factors compared in context to HUCPVC-hepatocyte contact cocultures. In transwell cocultures soluble factor gradients renders their stimulative concentration to lower levels than in direct coculture (310). Thus, it is possible that a HUCPVC secreted factor at high stimulative concentration blocks the beneficial effects that HUCPVCs may have on the increase in
CYP activity. This speculation is supported by the diminishment in CYP activity correlated with an increase in the concentration of HUCPVC CM (Figure 14). On the other hand, in cultures with flash frozen HUCPVCs there are three non-soluble factors that are missing from the live HUCPVCs: proliferation, migration, adaptation and membrane protein renewal. Since the lack of proliferation does not show a significant improvement in CYP activity (Figure 16), it indicates that this is not the cause. Adaptation, membrane protein renewal, and migration might not play a negative role either, as in every study, the CYP activity had an increase after 3 days of culture, during which HUCPVCs would have had some adaptation, membrane protein renewal and associated migration. Furthermore, we tested whether there was any significant difference between flash frozen HUCPVCs that were previously cultured alone and those that were exposed to hepatocyte soluble factors for 6 days (data not shown). The results indicated that adaptation due to soluble factors did not change any of the effects of the flash frozen HUCPVCs. Therefore, it appears that a HUCPVC soluble factor that requires a high concentration for its effects has a modulatory or negative effect in CYP activity. It is important to note however, that it is unknown whether this effect means a less mature CYP phenotype, as the expression of CYP families, specially the CYP3A family, varies during development changing the ratios of expression of the genes within this family (264, 311-313). Thus, hepatocyte immaturity does not necessarily mean less but rather higher CYP3A activity in some assays (23, 264, 313).

Interestingly while HUCPVCs do adapt to hepatocytes as shown in Chapter 3, we found that the medium in which HUCPVCs were cultured played a very important role in the secretion of the required hepatotrophic factors. The CM acquired from HUCPVCs cultured in their normal growth medium (Appendix 3) showed no effect on hepatocytes as opposed to the CM acquired from HUCPVCs cultured for at least 5 days in hepatocyte culture medium. This suggests that for a given environment, HUCPVCs adapt and acquire a trophic phenotype that can be significantly different from other conditions; in fact, using data acquired in Chapter 3, we observed that there was a significant difference (p <0.05) in the expression of laminin (2.82 fold in M1 relative to M2) and IL6 (<0.13 fold in M1 relative to M2) in HUCPVCs cultured alone in the two types of media used in this project. Nevertheless, hepatocytes have significant effects on the levels of some
hepatotrophic factors expressed by HUCPVCs (Chapter 3). However, when we tested whether there was any difference in the effect of naive or previously hepatocyte conditioned (HC) HUCPVC CM, flash frozen HUCPVCs and HUCPVCs, we found no significant difference in their interactions with hepatocytes. Furthermore, the initial lag phase in the manifestation of the positive effects in albumin secretion was almost eliminated with naive HUCPVC CM at high concentrations, indicating that the delay in upregulation of soluble hepatotrophic factors is partly the cause for the lag phase. Thus, the reason why the CM produced from naive and HC HUCPVCs did not show any difference could be because at the high (5X) and normal (1X) concentrations used, the changes generated by the HC HUCPVC CM were already obscured by having both CMs crossing above or below the threshold for manifesting differential effects.

5.4. HUCPVC trophic characteristics differ from that of BM-MSCs

As previously shown, the fact that BM-MSCs had a hepatotrophic effect led to the formulation of our hypothesis. Thus, in order to have a better understanding of the hepatotrophic level of HUCPVCs, we compared them with BM-MSCs. Initially, we used rat BM-MSCs and observed that their hepatotrophic phenotype while present was less consistent than that of HUCPVCs. In fact, it was not until we used the conditions from Chapters 2 and 4, that we actually saw their hepatotrophic phenotype (Appendix 5), as we were unable to see any using the culture conditions from Chapter 3. Thus from our observations HUCPVCs appear to be much more amenable than rBM-MSCs. Interestingly, this observation also suggests that hepatotrophic interactions appear to be species independent and at least hepatocytes do not show a preference towards allogeneic cells. This is further supported by the reciprocal effect that rat hepatocytes had on HUCPVCs, indicating that most, if not all crosstalk occurs in xenogeneic cultures. Furthermore, various studies have shown the benefit of human stromal cells and other species on rat hepatocytes (114, 225, 228, 309). Consequently, HUCPVCs could potentially support porcine hepatocytes, which are the cells being used in most BALS
(Table 2). HUCPVCs, unlike porcine umbilical cord perivascular cells, would have the advantage of being allogeneic thus being able to better react to the inflammatory environment of patients, a potentially important aspect for liver therapy as mentioned in Chapter 4.

Nevertheless, we considered that the comparison between rBM-MSCs and HUCPVCs was not appropriate, as human BM-MSCs have different characteristics to rBM-MSCs: slower proliferation, less prone to differentiation, faster senescence (314). Furthermore, rat dermal fibroblasts have shown considerable difference, in their level of support of rat hepatocytes, compared to human dermal fibroblasts (225). Additionally since we aimed to compare HUCPVCs to BM-MSCs in relation with their potential as liver therapeutics, it was necessary to employ same species MSCs; immunomodulation is important for liver therapy and much difference has been shown in the way different species MSCs deal with inflammation (284). Furthermore, it was unknown how human BM-MSCs would behave with hepatocytes, as all the previous research with BM-MSC-hepatocyte cocultures has been allogeneic or autologous using rat or pig cells. Interestingly, a comparison of the data acquired from hepatocyte cocultures with hBM-MSCs and rBM-MSCs, shows different levels on the effect on hepatocytes. Using HUCPVCs as the reference cell type, we can see that hBM-MSCs are able to increase albumin secretion and CYP activity much more than rBM-MSCs (Appendix 5, Chapter 4). Thus if we consider the difference in albumin levels, we can suggest that unlike dermal fibroblasts (225), hBM-MSCs have an improved hepatotrophic phenotype compared to rBM-MSCs; the same asseveration can also be made for HUCPVCs. Thus, both HUCPVCs and hBM-MSCs were able to significantly improve albumin secretion, ureagenesis, the expression of hepato-specific genes and allow net increase in mature hepatocyte mass. However, while the tendency for HUCPVCs was to have a faster improvement in ureagenesis, hBM-MSCs induced a significant increase in CYP activity.

Different types of fibroblasts have shown different levels of hepatotrophic capacity (87, 114, 225, 228, 242). Most of the tested ones are murine derived and are not primary but immortalized cell lines (87, 114, 228, 242). There are important advantages on using immortalized cell lines: consistency, characterization, and virtually infinite availability.
However a major drawback is the fact that being immortal the risk of oncogeny is considerably increased; and if being xenogeneic, there are also immunogeneic issues (17, 21). Neither HUCPVCs nor BM-MSCs have an unlimited capacity to proliferate (315), thus significantly reducing any oncogeny. They are allogeneic and importantly have been proven to be hepatotrophic, non-alloreactive and immunomodulatory (65, 96, 97, 196). The disadvantage of variability between donors, due in part to being heterogeneous populations can be overcome by pooling these cells, due to the non-alloreactive phenotype, while increasing the overall stability between therapeutic doses. In addition, the CFU-F frequency at harvest of HUCPVCs is orders of magnitude higher than that of BM-MSCs (94, 203), and their proliferation rate is faster (202). It might be argued that human dermal fibroblasts (225) can be hepatotrophic while also presenting MSC characteristics (316, 317). While this point is true, their multipotentiality can indicate that there was a preferential expansion of perivascular cells, the site where MSCs reside in every body site (186). Additionally, much controversy exists around the phenotype of dermal fibroblasts: they have been shown to be mutipotential but not immunoregulatory (318); or to immunoregulate without any differentiation capacity (319, 320). These discrepancies can be explained by the high degree of fibroblast heterogeneity that exists in the skin, leading to the possible isolation of multiple types of mesenchymal populations. Therefore, while the skin is easily accessible and available from numerous foreskins, the heterogeneity makes them a potential threat after culture as, unlike HUCPVCs or BM-MSCs (321), it has been possible to acquire immortalized cell lines from fibroblast harvests. Additionally, while every new born discards an umbilical cord, not every new born is circumcised; thus the availability of HUCPVCs is far higher than dermal fibroblasts while being less heterogeneous, allowing for the more frequent acquisition of hepatotrophic populations.

As mentioned in Chapter 4, both HUCPVCs and BM-MSCs lack telomerase activity and additionally have a similar expression for factors that can aid in wound healing. With respect to the liver, BM-MSCs have specifically being shown to be antifibrotic and anti-inflammatory. Interestingly the antifibrotic characteristics are linked to the anti-inflammatory phenotype of MSCs. In vivo, such a relationship is obvious, as chronic inflammation leads to chronic injury, which later leads to fibrosis; thus inhibition of
inflammation indirectly inhibits fibrosis. But antifibrotic effects have been shown to be independent of their anti-inflammatory effect by Parekkadan et al (161). They showed that BM-MSCs were able to modulate SC activation through soluble factors. The modulation reduced the collagen secretion and proliferation of SCs and induced apoptosis, indicating that BM-MSCs were directly antifibrotic. The BM-MSC factors that were found to be crucial in the modulation included HGF, TNFα and IL10. While we do not yet know how HUCPVCs would react in the presence of SCs, we do know that HUCPVCs have a lower expression of HGF in basal conditions than BM-MSCs, but coculture with hepatocytes increases it significantly, making it more than 6 fold the expression of BM-MSCs in coculture. Thus, HGF secretion, which was found to be associated with apoptosis of activated SCs, can indeed be produced by HUCPVCs at considerable levels. TNFα and IL10 were seen similarly expressed between the two MSCs. However, while IL10 expression has been constitutively seen in BM-MSCs (97, 322), it may not always be so (161, 278), thus a similar expression from the microarray data could also have meant its absence in both MSCs. While contact between immune cells and MSCs has been suggested to be required for IL10 upregulation (323); Parekkadan et al (161) saw an increase in IL10 production upon stimulation with IL6. IL6 mediated activation requires the presence of IL6-R and GP130 and we found no significant difference (p<0.001 and 1.5<fold difference) in the expression of these genes in HUCPVCs relative to BM-MSCs; indicating that IL6 stimulation would have similar potency in both cell types and probably also a similar increase in IL10. Thus, it is highly possible that HUCPVCs also have a direct modulatory effect on SCs.

The anti-inflammatory effect in vitro has been previously shown in our group (196) to be similar between the two MSC types. It was also suggested that soluble factors were the mediators of the interaction. While this may be true, non-soluble factors cannot be excluded, as it has been previously shown by Augello et al. (277), that MSCs may adapt to the lack of contact upregulating soluble anti-inflammatory factors. Among the soluble anti-inflammatory factors, IL10 has been suggested to be implicated in the MSC-lymphocyte interaction. While we are not sure of its true expression in HUCPVCs, we did not focus much on it due to the current controversy on whether MSC IL10 secretion or MSC IL10 induction in monocytes is the important mediator of immunomodulation.
Additionally, while the levels of IL10 produced by hBM-MSCs can abrogate lymphocyte proliferation, its blockage using antibodies causes no negative effects on the soluble anti-inflammatory effect (322). On the other hand, the effect of IDO has been shown to be directly linked to hBM-MSC immunosuppression (284, 322). Also, IDO is implicated in the induction of the secretion of IL10 in immune cells (324, 325). Our findings showed that it was produced in a higher amount in hBM-MSCs after induction using IFN-γ. This may indicate that BM-MSCs have an advantage over HUCPVCs in non-contact conditions, as HUCPVCs have a higher expression of PD1 ligands. The PD1 ligands, PD-L1 and PD-L2 are usually found in the cell membrane, thus being preferentially a contact factor, although not exclusive (295). The effects of the two factors have been shown to be crucial by Augello et al, but PD-L1 has been more extensively investigated (326) and even shown to be, along with IDO, important for the immuneprivileged of human umbilical cord derived MSCs (282). Also, PD-L1 stimulates the development and suppressive function of regulatory T cells (327). The p38 MAPK pathway has shown to positively influence the expression of PD-L1 (328), and we found that MAPK13, of the p38 MAPK, had a higher expression in HUCPVCs (1.83 fold of BM-MSCs, p<0.005), perhaps explaining the higher expression of this ligand in HUCPVCs. The expression of the PD-L2 had been shown to be lower in NF-KB p50/-/p65+/- mice (329) indicating that both NFKB1 and RELA components are important for its expression. In HUCPVCs the expression of RELA did not show difference from BM-MSCs but that of NFKB1 was 1.65 fold (p<0.005). Furthermore, the transcription factor OCT2 also had a higher expression, 1.57 fold of BM-MSCs’ (p<0.005). OCT2 expression has been shown to be responsible for the constitutive expression of PD-L2 in B-1 cells (330), and it is possible that it is also involved with the higher expression of this ligand in HUCPVCs. To date, it has been suggested that PD-L1 is involved in the regulation of autoreactive T cells thus inducing self-tolerance, while PD-L2 has to do with the regulation of T cell response to external antigens (331, 332). However, PD-L1 has received much attention in regard to its implications in transplantation, due to the positive correlation that it has shown with graft acceptance (283, 333).
5.5. Conclusions

From the studies presented herein in can be concluded that:

- HUCPVC provide hepatocytes with required signals for the maintenance of their mature phenotype in vitro: promoting net hepatocyte expansion, improving the maintenance of albumin secretion, ureagenesis, functional polarity and hepatotrope-related gene expression. Both soluble and non-soluble factors play different roles in the support.
- HUCPVCs are also influenced by hepatocytes, upregulating the levels of at least two hepatotrophic factors.
- HUCPVCs and BM-MSCs have different effects on hepatocytes: HUCPVCs have a faster effect on the maintenance of ureagenesis while BM-MSCs increase CYP activity.
- HUCPVCs and BM-MSCs have different levels of expression of hepatotrophic factors both constitutively and in coculture with hepatocytes. In addition, the expression of anti-inflammatory factors differs between the two cell types. Specifically HUCPVCs have a stronger expression of PD-1 ligands and BM-MSCs secrete higher amounts of IDO.

5.6. Future Perspectives

From the studies performed in this work, five extension studies can be proposed: Further improvement of coculture conditions; investigation of the effects of HUCPVC-hepatocyte liver assist devices in liver failure; study of the effects of a HUCPVC liver assist device and HUCPVC soluble factors in liver inflammation or acute liver failure; and the exploration of the effects of cotransplantation of HUCPVCs with liver allografts.

As previously mentioned the HUCPVC-hepatocyte coculture provides various improvements to the hepatocyte overall maintenance of its phenotype, however full recovery of ureagenesis is still elusive. We have already suggested that it is possible that the missing endothelial factors could be the cause of the inability of hepatocytes to fully
recover their original capacity. However, there are many other factors that can still be worked on to improve the conditions of the system. As shown in Chapter 3, medium formulation has a significant effect on the coculture system, thus improved formulations could further improve the system; for instance, the variation of nicotinamide concentration can lead to the re-expression of IL6 by HUCPVCs, thus finding a balance between the levels of nicotinamide and IL6 expression could lead to improved ureagenesis and albumin secretion (119, 230). Furthermore, since Matrigel and the collagen sandwich culture configuration can provide benefits to hepatocytes on their own, they could also enhance the coculture system. Since we observed, in Chapter 2, that nonsoluble and soluble factors independently had a higher increase in CYP activity than HUCPVCs in direct contact, it means that HUCPVCs have a constitutive capacity to further increase this activity. We also witnessed that at a very high HUCPVC confluence hepatocytes would lose their CYP activity. At a very high HUCPVC confluence, we think that tensile forces between cells increase, as we have been able to observe by the eventual roll-off of the cells sheet; and in the conditions used to test soluble and nonsoluble factors, tensile forces coming from HUCPVCS would not exist. From these, it can be suggested that tensile forces are causing a change in CYP activity. Thus it is possible that HUCPVCs with downregulated smooth muscle actin could improve CYP activity. Another aspect that can be investigated is the control of tissue architecture, thus controlling the amount of interaction between HUCPVCs and hepatocytes. This approach would allow the isolation of factors such as tensile forces, cell-cell contact ratio, migration and cell concentration ratio. This could be done by means of patterned surfaces similarly to the way described by Bathia et al (334, 335).

Another branch of research would be the actual test of HUCPVC-hepatocyte cocultures in liver illness models. An approach similar to the one used by Yagi et al (242) could be used, where initially the cultures were exposed to serum from ALF-rats to confirm the robustness of the coculture, and later a flat plate bioreactor could be tested on the ALF-rats and determine the improvement in survival. In order to test the effects of coculture in cirrhosis, the use of analbuminemic rats would be recommended, due to their total absence of serum albumin. In this case the first step would be to implant coencapsulated HUCPVCs and hepatocytes into the peritoneum and compare the albumin output with
encapsulated hepatocytes. If cocultures showed a higher albumin output, cirrhosis could be induced in the analbuminemic rats (according to the protocol in Appendix 1) and the rats could be treated with the encapsulated cocultures and monocultures, again the albumin output would indicate that the implanted hepatocytes are actually rescuing the subject.

Additionally, HUCPVCs could be used on their own for the treatment of liver injury or failure. As shown by Parekkadan et al (154) using BM-MSCs, HUCPVCs CM and lysates could be directly tested as a therapeutic tool by intravenous injection to treat ALF. A different approach also showed by Parekkadan et al (154) was the use of an extracorporeal device to which the subject’s plasma was exposed to allow the soluble factors to be released into the blood stream and treat liver failure. Other authors also have tested the use of MSCs on other liver injury models by intravenous injection (247, 248, 250, 336), however due to the possibility of cell sequestering in the alveolar capillary bed, the best approach in this context would be HUCPVC injection into the portal vein as employed by Kharaziha et al (251). Another possible way to test whether HUCPVCs on their own would contribute to liver healing would be by their insertion into the Gilson’s capsule. This method might prove to be very useful for HUCPVCs as Tsai et al (279) used this approach with an MSC type, that may even contain HUCPVC populations, as it also came from the Wharton’s Jelly.

Finally, HUCPVCs could be used as adjuvants in liver transplantation. Their anti-inflammatory properties could aid in the process of tolerance to the allogeneic insult. Specifically, the higher expression of PD-L1 makes HUCPVCs a potentially highly effective cell, as PD-L1 has shown to be required for allograft tolerance (337). Furthermore, BM-MSCs have been shown to alleviate acute immune rejection of liver allografts (338). It is possible to use this approach, as Hing et al did, injecting HUCPVCs into the bloodstream and allow them to home into the liver, however cell sequestering in the lungs can again become a problem. A more direct approach could be the perfusion of liver allografts with HUCPVC containing fluid before transplantation, thus allowing for the instant action of HUCPVCs in the liver. The presence of HUCPVCs can have the advantage of both diminished rejection, as seen by Hong et al with BM-MSCs, and
protecting hepatocytes from injury due to their hepatotrophic phenotype. Furthermore, if HUCPVC grafting is successful it could even diminish the need for a constant intake of immunosuppressants by the recipient. While this statement might appear too optimistic, it is possible due to the fact that the liver is a relatively immuneprivileged organ and even in some human liver recipients complete withdrawal of immunosuppressants has been possible (339).
References


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Appendices

Appendix 1: Cirrhosis and Induction Protocol

A solution of CCl4 in corn oil was administered to Wistar rats by gavage weekly for 12 weeks on a full stomach around 1 pm. The solution concentration used was 32% during the whole trial. The volume of CCl4 solution started at 0.442 ml/kg of CCl4 and was later calculated according to the algorithm presented in Table 1. Phenobarbital was also administered through the drinking water since two weeks before starting the gavage of CCl4 and during the treatment, at a dose 0.35 g/l ad libitum to reinforce the liver injury (118, 340-342). Rats were weigh at least three times a week and blood samples were taken weekly, just before gavage, under anaesthesia (with Isofluorane in nitrous oxide and oxygen, 3% induction and 1.5% maintenance).

Table 1: Any increase in dose will only happen if there is increase in weight after one week, otherwise the last dose would be used.

<table>
<thead>
<tr>
<th>Maximum weight decrease after CCl4 treatment (percentage)</th>
<th>Dose administered (X=initial dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;3%</td>
<td>Last dose +X</td>
</tr>
<tr>
<td>3-6%</td>
<td>Last dose +0.75X</td>
</tr>
<tr>
<td>6-9%</td>
<td>Last dose +0.5X</td>
</tr>
<tr>
<td>9-11%</td>
<td>Last dose</td>
</tr>
<tr>
<td>11-12%</td>
<td>Last dose -0.5X</td>
</tr>
<tr>
<td>&gt;12%</td>
<td>Last dose/2</td>
</tr>
</tbody>
</table>

During this treatment, there was no mortality and all of the rats behaved normally. The decrease of serum albumin levels to at least 20% of normal conditions was reached on all the rats treated without any incidence of inhabilitating ascites (see Table 2, Fig. 1C).

Table 2: Decrease albumin levels at 12 week of treatment in relation with normal levels (1-4 week)

| Rat 1 treated     | 38%     |
| Rat 2 treated     | 21%     |
| Rat 3 treated     | 32%     |
| Rat 4 Control     | 0.08%   |
| Rat 5 Control     | 1.51%   |
| Rat 6 Control     | -1.60%  |
Figure 1: Changes during treatment.
The numbers stand for the 3 subjects used. The control weight comes from the average of 3 rats only submitted to phenobarbital water ad libitum. A) Rats’ weight variation through the course of the treatment. B) CCl₄ dose used through the course of the treatment. C) Albumin levels used through the course of the treatment. D) Sorbitol dehydrogenase (SDH) serum levels during the course of the treatment.

Figure 2: Rat Liver slices stained with picrosirius red.
A), C) healthy liver. B), D) cirrhotic liver. A), B) pictures taken under direct light. C), D) under polarized light. FW: big picture, 1.4 mm; small picture, 5.4 mm.
Appendix 2: Additional Immunofluorescence characterization of HUCPVCs

Antibodies used:

Vimentin: 1:200. Labvision: RM9120-S.

Figure 1: Expression of cytokeratin 8 and 18 in HUCPVCs at passage 1 and 8. FW: 1.2 mm.

Figure 2: Intracellular expression of CK19. FW: 1.2 mm.

Figure 3: Localization of vimentin, CK19 and CK18 within an umbilical cord section. Note the vessel wall and the close perivascular area having reaction for CK18 and 19 while vimentin is ubiquitous. FW: 1.2 mm.
Appendix 3: Correlation of albumin transcript to albumin secretion. HUCPVC CM produced from HUCPVCs cultured with growth medium.

Figure 1: Albumin levels in coculture and with conditioned media. COCUL=Coculture; MONO=monoculture; 10x, 2x, 0.4x, 0.08x= Concentration of HUCPVC conditioned medium (CM) used in monoculture. A) Albumin protein secretion levels (MONO, COCUL) and albumin gene expression (MONOrna, COCULrna) relative to day 1. B) Hepatocytes were cultured in hepatocyte culture medium (HCM) or standard medium plus CM at different concentrations. As described in chapter 2 or 4, HCM is DMEM supplemented with 10% FBS, 1 µM Dexamethasone, 1x ITS, 100 u/ml Penicillin and 100 µg/ml. HUCPVC CM was produced as described in Chapter 2, however HUCPVCs were cultured only in HUCPVC growth medium: α-MEM supplemented with 10% FBS and antibiotics: penicillin G 167 U/mL, amphotericin B 0.3 mg/mL, gentamicin 50 mg/mL.
Appendix 4: Coculture compared to collagen sandwich monoculture.

Figure 1:
S-monoculture= Collagen Sandwich monoculture. A) High density culture of hepatocytes ($118.4 \times 10^3$ cells/cm$^2$). B) Medium density culture of hepatocytes ($39.5 \times 10^3$ cells/cm$^2$). C) Low density culture of hepatocytes ($13.2 \times 10^3$ cell/cm$^2$). Right: Albumin production rate of hepatocytes over time. Normalized to the initial seeding density of hepatocytes and day 1. Left pictures, HUCPVCs (green cells), hepatocytes (only brightfield). Cocultures compared to S-monocultures at two different time points. Top: day 2; Bottom: day 10. Field width: 2228 um. 4 sample replicates (N=4) for S-monoculture and 12 sample replicates (N=12) for coculture. Error bars indicate standard error. GEE analysis used to identify significant differences in data sets. * P<0.01, x<0.05.

Figure 2: Albumin production rate of hepatocytes over time.
Error bars indicate standard error. GEE analysis used to identify significant differences in data sets. Wilkoxon-Rank test at each time point to compare Coculture with Collagen Sandwich Monoculture (S-Monoculture)* P<0.01. N=12.
Appendix 5: rat BM-MSC cocultures.

Figure 1: Lack of effect of rBM-MSCs or fibroblasts on albumin secretion rate using the conditions described in Chapter 3; COL-I coated plates in Williams E medium supplemented with 2% FBS, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 50 ng/ml Gentamicin, 10 ng/ml EGF, 1mM ascorbic acid 2-P, 20 mM HEPES, 0.1 U/ml Insulin, 10^{-7} M Dexamethasone and 10mM Nicotinamide; since day 4 of culture 1% DMSO was also added to the culture medium. Hepatocytes cultured alone (Monoculture) or with rBM-MSCs (BMcocul) or OP-9 murine fibroblasts (Fib-cocul). Albumin levels relative to levels on day 2.

Figure 2: Hepatocytes cultured alone (MONO) or with rBM-MSCs (BMcocul) or HUCPVCs (HUCCcocul). Culture medium used was HCM, described in Chapter 2 or 4. BMcocul = grey bar; HUCCcocul= black bar; and MONO= white bar A) Hepatocyte albumin secretion over time, expressed relative to day 1 levels. B) Urea in medium relative to day 1. C) CYP activity relative to day 1. Error bars indicate standard Error. Wilcoxon Rank-sum test used compared to monoculture. In A), differences between cultures systems analysed with GEE. Data represents quantification from 3 biological replicates. *P<0.01, xP<0.05.
Appendix 6: Jagged1 expression is directly proportional to cell density

Figure 1: Semi-quantitative PCR of JAG1 expression in HUCVPVCs in relation with cell density. C stands for number of cycles used for end-point RT-PCR. HUVECs used as positive control. GAPDH: housekeeping gene.