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Evaluation of the anti-fibrotic properties of Adult-Derived Human Liver Stem/ progenitor Cells

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Abbreviations

ACEi: Angiotensin Converting Enzyme inhibitors

ADHLSC: Adult-Derived Human Liver Stem Cells

Alb: Albumin

ARB: Angiotensin Receptor 1 Blocker

ASMA: Alpha-Smooth Muscle Actin

BM-MSC: Bone Marrow-derived Mesenchymal Stem Cells

CCK-8: Cell Counting Kit-8

CCL: Chemokine (C-C motif) Ligand

CCl4: Carbon Tetrachloride

CK: Cytokeratin

CTGF : Connective Tissue Growth Factor

CXCL : Chemokine (CX-C motif) Ligand

CXCR4: Chemokine Receptor Type 4

DAB: Diaminobenzidine

EASL: European Association for the Study of the Liver

ECM: Extracellular matrix

EMT: Epithelial Mesenchymal Transition

ERK: Extracellular signal-Regulated Kinase

ESC: Embryonic Stem Cells

FGF: Fibroblast Growth Factor

GFP: Green Fluorescent Protein

GM-CSF: Granulocyte Macrophage Colony-Stimulating Factor

GSEA: Gene Set Enrichment Analysis

HBV: Hepatitis B Virus

HCV: Hepatitis C Virus

HGF: Hepatocyte Growth factor

HLA: Human Leukocyte Antigen

HSC: Hepatic Stellate Cell

ICAM-1: InterCellular Adhesion Molecule 1

IL: Interleukin

INF-ɣ: Interferon gamma

INR: International Normalized Ratio

IP: Intraperitoneal

iPSC: induced Pluripotent Stem Cells

LIF: Leukemia Inhibitory factor

LOXL2: Lysyl oxidase-like-2

LSEC: Liver Sinusoidal Endothelial Cells

MCD: Methionine-Choline-Deficient

MCP-1: Monocyte Chemoattractant Protein-1

MELD: Model for End-stage Liver Disease

MIP: Macrophage Inflammatory Protein

MMP: Matrix Metalloproteinase

MSC: Mesenchymal Stem Cell

NAC: N-Acetylcysteine

NASH: Non-Alcoholic SteatoHepatitis

NCAM: Neural Cell Adhesion Molecule

NF-κB: Nuclear factor Kappa B

NGF: Nerve Growth factor

NK cells: natural Killer Cells

OLT: Orthotopic Liver Transplantation

PBS: Phosphate Buffered Saline

PDGF: Platelet-Derived Growth Factor

PE: Phycoerythrin

PGE2: Prostaglandin E2

PI: Propridium Iodide

PPAR-ɤ: Peroxisome Proliferator-Activated Receptor gamma

PT: Prothrombin Time

RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted

RPM : Round Per Minute

SC: Subcutaneous

SCID: Severe Combined Immunodeficiency

SDF-1: Stromal cell-Derived Factor-1

SEM: Standard Error of the Mean

TBil: Total Bilirubin

TGFβ: Transforming Growth Factor Beta

TLR4: Toll-Like Receptor 4

TNF-α: Tumor Necrosis Factor- alpha

UDCA: Ursodeoxycholic Acid

VCAM-1: Vascular Cell Adhesion Molecule-1

VEGF: Vascular Endothelial Growth factor

Part I

Introduction

Liver fibrosis is a major health issue for which no effective treatment is available so far, except orthotopic liver transplantation (OLT). However, organ shortage is a daily reality. The efficacy of anti-fibrotic drugs has not been proven in humans. Hence, there is an urgent need to find alternative therapeutic strategies. Cell-based therapy may represent an attractive therapeutic option, based on the improvement of the hepatic inflammatory microenvironment, the inhibition of Hepatic Stellate Cell (HSC) activation or the induction of their apoptosis, the replacement of damaged hepatocytes and the promotion of residual hepatocytes regeneration. In particular, Mesenchymal Stem Cells (MSCs) represent an attractive therapeutic tool for the treatment of liver fibrosis, in terms of safety and efficacy.

In this part, we will try to make an overview of the current knowledge in terms of cell therapy using MSC in a context of liver fibrosis. We will then discuss the relevance of using MSC to treat this condition and highlight the future perspectives in this field.

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Authors: **Berardis Silvia**, Dwisthi Sattwika Prenali, Najimi Mustapha, Sokal Etienne Marc.

CHAPTER 1

Liver fibrosis

Progressive liver fibrosis is a major health issue for which no effective treatment is available so far, leading eventually to cirrhosis and orthotopic liver transplantation (OLT). However, organ shortage is a daily reality. Hence, there is an urgent need to find alternative therapeutic strategies. Mesenchymal Stem Cell-based therapy may represent an attractive therapeutic option, based on their immunomodulatory properties, their differentiation potential into hepatocytes allowing the replacement of damaged hepatocytes, their potential to promote the residual hepatocytes regeneration and their capacity to inhibit Hepatic Stellate Cell activation or to induce their apoptosis, in particular via paracrine mechanisms

In this part, we will highlight the recent knowledge regarding the input of MSC-based therapy for the treatment of liver fibrosis and will discuss the perspectives in this field.

1.1. Liver fibrosis: a major health issue

Liver fibrosis refers to the excessive accumulation of extracellular matrix into the liver parenchyma in response to chronic injury. Injuries may result from viral, autoimmune, cholestatic, toxic or metabolic disease, including nonalcoholic steatohepatitis. Chronic fibrosis progresses from fibrosis to cirrhosis characterized by septa formation and rings of scar surrounding nodules of surviving hepatocytes [1]. Epidemiological data suggest that cirrhosis affects hundreds of millions people around the world [1]. It represents the 14th most common cause of death in adults worldwide (resulting in 1.03 million death per year) but the fourth one in central Europe [2]. In the European population, less than 1% (~0.1%) is affected by cirrhosis which corresponds to 14-26 new cases per 100 000 inhabitants per year or an estimated 170 000 deaths per year [3].

1.2. Clinical aspects

While mild fibrosis remains largely asymptomatic, its progression towards cirrhosis is a major cause of morbidity and mortality. Fibrosis

and distorted vasculature lead to portal hypertension and related complications, namely upper gastrointestinal bleeding from ruptured gastroesophageal varices, portal hypertensive gastropathy, ascites, renal dysfunction, hypersplenism leading to thrombocytopenia and hepatopulmonary syndrome[4]. Furthermore, cirrhosis is associated with hepatocellular insufficiency , impaired metabolic capacity and dysfunction of other organs such as the gastrointestinal tract [5], the kidneys [6], as well as the cardiovascular [7], respiratory [8] and skeletal systems [9]. Cirrhosis can lead to hepatocellular carcinoma [10].

1.3. Histology of liver fibrosis

Following acute injury, liver parenchymal cells regenerate and replace the necrotic damaged cells. During this process, an inflammatory response is observed and accompanied by a limited deposition of extracellular matrix. In case of persistence of the injury, the regenerative capacity of parenchymal cells is impaired and dead hepatocytes are replaced by an abundant accumulation of the extracellular matrix, mainly secreted by activated hepatic stellate cells [11]. The initial distribution of this extracellular matrix will depend on

the etiology of liver injury. In chronic viral hepatitis and chronic cholestatic disorders, the fibrotic tissue will initially be located in the periportal areas. However, in alcohol-induced liver disease, the pericentral and perisinusoidal areas represent the initial localization of extracellular matrix deposition [12], probably because alcohol is mainly metabolized in these regions.

Together with disease progression, the collagen fibers will progressively progress to bridging fibrosis, leading finally to cirrhosis. In advanced stages of fibrosis, the liver contains approximately 6 times more extracellular matrix deposition levels than a normal liver. including collagens (types I, III and IV), fibronectin, undulin, elastin, laminin, hyaluronan and proteoglycans [11]. The accumulation of extracellular matrix into the liver parenchyma results from both an increased synthesis and a decreased degradation by matrix metalloproteinases.

Cirrhosis is defined histologically as a diffuse process characterized by fibrosis and the conversion of normal liver architecture into structurally abnormal nodules [13].

1.4. Physiopathology of liver fibrosis

1.4.1. Cellular effectors: extracellular matrix producing cells

Extracellular matrix is mainly produced by Hepatic Stellate Cells (HSC), located in the space of Disse between hepatocytes and sinusoids. Following liver injury, HSC are "activated" and evolve to myofibroblast-like cells following paracrine and autocrine signals. This activation is characterized by an increase in cell proliferation and extracellular matrix protein deposition, by a loss of the vitamin A droplets and by the acquisition of contractile features. Initiation represents the first activation phase and refers to early changes in gene expression and phenotype. HSC are stimulated by paracrine signals, including exposure to lipid peroxides and products released from damaged hepatocytes as well as biochemical signals from Kupffer and endothelial cells. In the perpetuation phase, the activated phenotype is maintained and fibrosis is generated. Autocrine as well as paracrine loops are implicated. Resolution refers either to the reversion to a quiescent phenotype or to a clearance through apoptosis [14]. At the structural level, activated HSC lose their big Vitamin A containing lipid droplets and up-regulate the expression of cell adhesion molecules like ICAM-1 and VCAM-1,

promoting the recruitment of inflammatory cells to the injured liver. The up-regulation of adhesion molecules expression has been studied in vitro and in vivo [15]. The expression of α-smooth muscle actin is also up-regulated and the secretion of pro-inflammatory cytokines is increased [16] [14].

During liver fibrosis development, HSC secrete matrix metalloproteinases (MMPs), calcium-dependant enzymes that specifically degrade collagens and noncollagenous substrates. The normal hepatic matrix is progressively disrupted and replaced by scar matrix, which will impair cell function. HSCs are the principal source of MMP2, MMP9 and MMP13 and stromelysin. Increased expression of MMP2 is characteristic of active fibrogenesis and is a key feature of HSC activation. Liver fibrosis is characterized by a failure to degrade the increased interstitial extracellular matrix, mainly composed of collagen type I. MMP1 is the main protease that can degrade collagen I. HSCs express MMP1 mRNA but little enzyme can be detected [14].

Beside HSC, other cellular sources contributing to extracellular matrix accumulation have been identified. These cells include portal fibroblasts (mainly implicated in biliary fibrosis) [17], circulating

fibrocytes, bone-marrow derived cells [18] as well as fibroblasts derived from epithelial-mesenchymal transition (EMT) of hepatocytes and bile duct epithelial cells [19]. EMT is characterized by a loss of cell adhesion, a repression of E-cadherin expression and an increased cell mobility. This phenomenon represents an attractive target for liver fibrosis treatment.

1.4.2. Other cellular sources involved in fibrogenesis

- Biliary progenitor cells

In biliary fibrosis, the proliferating biliary progenitor cells secrete several factors that attract and activate HSC into proliferative and extracellular matrix – producing cells. This phenomenon is amplified by several molecules secreted by the surrounding myofibroblasts and by inflammatory cells, like IL-6 and fibroblast growth factor [20].

- Liver Sinusoidal Endothelial Cells

In perisinusoidal fibrosis, the Liver Sinusoidal Endothelial Cells (LSEC) are activated and proliferate. LSEC contribute to extracellular matrix production; secrete cytokines and growth factors (such as TGFβ and

PDGF) that activate HSC as well as factors contributing to intrahepatic vasoconstriction. Myofibroblasts activate LSEC via secretion of angiogenic factors such as VEGF and angiopoietin-1 [21].

- Inflammatory cells

CD4+T cells with a Th2 polarization also promote fibrogenesis. These cells secrete IL-4 and IL-13 which can stimulate the differentiation of fibrogenic myeloid cells and macrophages [22]. Th17 cells, induced by TGF-β1 and IL-6, secrete IL-17A, which activates directly myofibroblasts and indirectly by stimulating TGF-β1 release by inflammatory cells [23]. Regulatory T cells can either favor or inhibit fibrogenesis by secreting TGF-β1 (profibrotic) or IL-10 (anti-fibrotic) [20]. CD4+ Th1 cells have an anti-fibrotic effect [20].

NK cells can reduce fibrosis by killing activated HSC and by producing interferon γ [24]. Monocytes play a key role in inflammation and fibrosis. They are precursor of fibrocytes, macrophages and dendritic cells [25]. Macrophages are fibrogenic during fibrosis progression and fibrolytic during its reversal [20].

1.4.3. Key factors

- Factors involved in HSC proliferation

PDGF-β signaling is one of the best characterized pathways involved in HSC activation process. After PDGF-β binding to its receptor, several intracellular pathways are activated (including Ras-MAPK, PI3K-AKT/PKB and PKC pathways) supporting cellular proliferation. In early HSC activation, a rapid induction of PDGF-β receptor is observed [26] [27].

Even if PDGF is the most potent mitogen towards HSC, other growth factors such as TGFα, EGF and VEGF are also able to stimulate HSC proliferation [28].

- Fibrogenic molecules

TGFβ1 derives from both autocrine and paracrine sources. It represents the most potent fibrogenic cytokine in the liver. TGFβ1 recruits Smad2/3, leading to their phosphorylation and stimulation of fibrogenic gene expression [29]. Leptin also has a pro-fibrotic action through suppression of peroxisome proliferator-activated receptor-ɤ

(PPARɤ) [30]. Connective tissue growth factor (CTGF), secreted by HSC, is also fibrogenic.

- Chemokines

The migration of HSC to the site of injury is promoted by several chemokines (such as CCL5) secreted by HSC which express the appropriate receptors [28].

- Neurotransmitters

Following chronic liver injury, the local neuroendocrine system is up-regulated and HSC express different receptors, including those regulating cannabinoid signaling, and secrete endogenous cannabinoid. The activation of CB1 receptor is pro-fibrogenic while CB2 receptor is anti-fibrotic. Opioid and serotonin pathways as well as thyroid hormones have a pro-fibrotic effect [28].

- Inflammatory pathways

Finally, inflammatory pathways are also involved in HSC activation process. HSC secrete inflammatory chemokines and interact directly

with immune cells through expression of adhesion molecules, including ICAM-1 and VCAM-1 [31]. Moreover, apoptotic hepatocyte DNA can interact with Toll like receptor 9 expressed on HSC, repressing HSC migration and increasing collagen production [32].

CHAPTER 2

Current therapeutic approaches

2.1. Anti-fibrotic drugs

Liver fibrosis is a dynamic process that may undergo reversal [33]. The best aim of anti-fibrotic therapy is to eliminate the underlying disease process. In case of impossibility to treat the underlying process, anti-fibrotic therapy would be ideal. Currently, there is no anti-fibrotic drug available in clinical setting [34] [1] [35]. Specific agents are under investigation. However, none has been approved as anti-fibrotic therapy.

The use of anti-fibrotic drugs has been reported in preclinical and clinical studies. This approach targets several aims[36] [37] [38], such as: (1) downregulate HSC activation [39] [40] [41] [42] [43] [44] [45] [46], (2) neutralize proliferative, fibrogenic, and contractile responses of HSC [47] [48] [49] [50] [51] [52] [53], (3) promote apoptosis of HSC [54] [55], (4) promote matrix degradation [56] [57], (5) reduce inflammation [58] [59] [60] [61] [62] [63] and (6) inhibit collagen I cross-linking [64], as

shown in Table 1. Overall, anti-fibrotic agents have been shown to be highly effective in animal models and represent potential anti-fibrotic drugs. Several anti-fibrotic agents that have been transitioned to clinical studies are peroxisomal proliferator-activated receptor gamma (PPAR-γ) agonist [40] [41], interferon γ (IFN-γ) [43] [44], angiotensin II antagonist [50], colchicine [52], interleukin 10 (IL-10) [59], anti-tumor necrosis factor alpha (TNF-α) [61], ursodeoxycolic acid [63], and antioxidant [46].

Given the supportive preclinical data, however, the data in human are mixed. Moreover, most of these studies are performed in small numbers of patients during a short period of time, whilst fibrosis is a long lasting, slowly progressive event. Human studies have examined the effect of PPAR-γ agonist [40] and IFN-γ [43] in patients with liver fibrosis. Beside promising results in small scale studies [40] [43], longer and larger studies failed to demonstrate any beneficial effect [41] [44].

Compared to preclinical studies, clinical studies of several anti-fibrotic agents have been shown to yield dramatically different results [46] [52] [59] that may be due to several reasons. In animal model, anti-fibrotic drugs were investigated against the development of fibrosis. On the

other hand, in real clinical setting, and in most of clinical trials, patients have advanced fibrosis. Potential of collagen degradation also differs between rodent model and human as a consequence of different cross-linking of ECM. Compared to human fibrosis, that requires years to develop, fibrosis in rodent takes place in weeks or months and contains less chemical cross linking. In addition, differences in pharmacokinetics of anti-fibrotic drugs between animal model and human contribute to the different results [37].

Furthermore, a crucial issue that remains to be investigated is how to translate preclinical evidences of other potential anti-fibrotic agents into benefit for patients. In general, the development of anti-fibrotic drugs in human meets several obstacles [36]. First, liver fibrosis is slowly progressive event, requiring probably several years of follow up to establish efficacy. Second, gold standard tool to evaluate fibrosis remains histology. Patients and physicians may be reluctant to perform repeated biopsies, due to possible adverse events [65]. Moreover, sampling error in liver biopsy and inter-observer variability may interfere with the results [66]. For all these reasons, noninvasive diagnostic tools would be highly desirable, ranging from physical

examination finding, laboratory investigation, radiographic test, to specific serum marker [37]. Transient elastography has also been developed to measure liver stiffness using ultrasound principle [67].

Table 1: Preclinical and clinical studies representing the development of anti-fibrotic strategies

Antifibrotic drug	Preclinical/ clinical results	Disease model	Reference
Downregulation of HSC activation			
Peroxisomal proliferator-activated receptor gamma (PPAR-γ) agonist (Pioglitazone)	Inhibition of HSC activation and amelioration of hepatocyte necroinflammation in rats after 8 weeks	Carbon tetrachloride (CCl4)-induced liver fibrosis	[39]
	Reduction of steatosis, but not fibrosis compared to placebo, in patients with NASH after 6 months (26 pioglitazone; 21 placebo)	Nonalcoholic steatohepatitis (NASH)	[40]
	No benefit of pioglitazone over placebo in term of steatosis and fibrosis in patients with NASH after 96 weeks (80 pioglitazone; 83 placebo)	Nonalcoholic steatohepatitis (NASH)	[41]
Interferon gamma (IFN-γ)	Inhibition of the activation of HSC and extracellular matrix production	Carbon tetrachloride (CCl4)-induced liver fibrosis	[42]
	Improvement of fibrosis scores in patients with chronic HBV infection after 9 months (54 IFN-γ; 29 control)	Chronic HBV infection	[43]
	No reversion of fibrosis in patients with advanced liver disease after 1 year (IFN-γ1b 100µg 169; IFN-γ1b 200µg 157; placebo 162)	Chronic HCV infection	[44]
Antioxidant (Vitamin E)	Protective effects against liver damage and cirrhosis in rats	Carbon tetrachloride (CCl4)-induced liver fibrosis	[45]
	No benefit on liver function tests in patients with mild to moderate alcoholic hepatitis after 1 year (25 vitamin E, 26 placebo)	Alcoholic hepatitis	[46]
Neutralization of proliferative, fibrogenic, and contractile responses of HSC			
Anti-Transforming Growth Factor beta (TGF-β)	Supression of fibrosis in rats after 3 weeks	Dimethylnitrosamine-induced liver fibrosis	[47]
Short interference RNA (SiRNA)	Inhibition of the expression of TGF-β1 and attenuation of liver fibrosis in rats	High-fat diet and CCl4-induced model of liver fibrosis	[53]
Endothelin antagonist	Nonpeptide endothelin-A receptor antagonist, LU 135252, reduced collagen accumulation in rats after 6 weeks	Secondary biliary fibrosis	[48]
Angiotensin system inhibitor	Olmesartan, an angiotensin II type 1 receptor blocker, decreased expression of collagen genes and attenuated liver fibrosis in rats after 15 weeks	Methionine-choline-deficient rat model of NASH	[49]
	Angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor-1 blocker (ARB) did not retard the progression of liver fibrosis in patients with advanced liver fibrosis after 3.5 years (66 ACEi/ARB, 126 non-ACEi/ARB, 343 no antihypertensive medication)	Chronic hepatitis C	[50]
Colchicine	Colchicine and colchiceine (metabolite of colchicine) prevented increase in collagen synthesis and increased the intracellular degradation of collagen rats	Carbon tetrachloride (CCl4)-induced liver fibrosis	[51]
	Colchicine improved fibrosis marker, but not histological finding, in patients with hepatic fibrosis after 12 months (21 colchicine; 17 control)	Liver fibrosis of various etiologies	[52]
Promotion of HSC apoptosis			
Gliotoxin	Morphologic alterations typical of apoptosis of HSC in vitro (activated rat and human HSC) and reduction of the number of activated HSC in rats	Carbon tetrachloride (CCl4)-induced liver fibrosis	[54]
Sulfasalazine	Induction of activated HSC apoptosis, by inhibiting nuclear factor kappa B-dependent gene transcription, both in vitro (activated rat and human HSC) and in vivo	Carbon tetrachloride (CCl4)-induced liver fibrosis	[55]

Promotion of matrix degradation				
	Matrix metalloproteinase (MMP) inducer	Urokinase-type plasminogen activator, an initiator of the matrix proteolysis cascade, induced collagenase expression and reversal of fibrosis rats	Carbon tetrachloride (CCl4)-induced liver fibrosis	[56]
	Tissue inhibitor of matrix metalloproteinase (TIMP) inhibitor	Polaprezinc, a zinc-carnosine chelate compound, attenuated fibrosis by inhibiting TIMP expression during a later phase, thus promoting fibrinolysis, in mice after 10 weeks	Dietary methionine and choline deficient (MCD)-induced NASH	[57]
Reduce inflammation				
	Interleukin 10 (IL-10)	Inhibition of HSC activation and decrease of the expression of TGF-β1, MMP-2, and TIMP-1 in rats	Carbon tetrachloride (CCl4)-induced liver fibrosis	[58]
		Anti-inflammatory effect, but increased HCV viral burden via alterations in immunologic viral surveillance, in patients (30 subjects for 3-dose trial)	Chronic hepatitis C	[59]
	Anti-TNF-α	Infliximab decreased necrosis, inflammation, and fibrosis in rats	Dietary methionine and choline deficient (MCD)-induced NASH	[60]
		Infliximab improved Maddrey's score in patients after 28 days (20 subjects)	Alcoholic hepatitis	[61]
	Ursodeoxycholic acid (UDCA)	Reversion of liver damage in rats	Carbon tetrachloride (CCl4)-induced liver fibrosis	[62]
		Reduction of periportal necroinflammation and, if initiated at the earlier stages I-II of the disease, delay of the progression of histologic stage in patients after 2 years (200 UDCA, 167 placebo)	Primary biliary cirrhosis	[63]
Inhibition of collagen I cross-linking				
	Anti-Lysyl oxidase-like-2 (LOXL2)	Reduction of liver fibrosis, decrease in the number of myofibroblasts and lower p-Smad3 signal	Carbon tetrachloride (CCl4)-induced liver fibrosis	[64]

2.2. Orthotopic liver transplantation

Currently, orthotopic liver transplantation (OLT) remains the most effective treatment for this condition. Over time, the survival rate after OLT has progressively increased, reaching currently 83% after one year in adults. Liver cirrhosis remains the main indication for OLT in Europe (59%) (EASL 2013). In children, a survival rate of 93.2% has been

reported 10 years after OLT [68]. However, over the last 10 years, the annual number of OLT has stopped growing because organ donation has not kept up with demand, leading to an increased mortality and morbidity in the waiting list [69]. Moreover, some limitations such as operative risk, post-transplant rejection, recurrence of the pre-existing liver disease and high costs have to be taken into account [70]. Moreover, fibrosis often develops in the liver grafts as early as one year after transplantation. Portal fibrosis is present in 31% of liver grafts one year after pediatric OLT [71] and in 74% in the long term [72] [73]. The prevalence increases to 65% five years after OLT and to 71% at 10 years, with 29% of severe fibrosis [74].

2.3. Cell-based therapy

Cell-based therapy has been proposed as a less invasive potential alternative to OLT. The rationale is mainly based on the ability of several cells to (1) improve the hepatic inflammatory microenvironment, (2) inhibit the activation or induce apoptosis of HSC, (3) replace damaged hepatocytes and (4) promote the regeneration of residual hepatocytes.

- *Isolated hepatocytes*

Hepatocyte transplantation has provided the proof-of-concept that cell therapy could be used to treat some liver diseases such as metabolic disorders and acute liver failure [75] [76] [77]. A decrease of liver fibrosis and a restoration of phospholipid secretion were also observed in a mouse model of progressive familial intrahepatic cholestasis type III after hepatocyte transplantation [78]. The feasibility and safety of this technique is supported by the numerous clinical trials performed with hepatocytes.

However, the efficacy seemed to have a limited durability, with a progressive decrease of the observed effects [79]. Moreover, hepatocytes are poorly resistant to cryopreservation, which can be limitative as fresh hepatocytes are not always available [80]. Moreover, hepatocytes are rare material and cannot be expanded in vitro. Therefore, finding a new and readily available cell source was primordial.

- *Stem/progenitor cells*

Stem/progenitor cells have progressively emerged as an attractive alternative to hepatocytes in the context of cell-based therapy. Stem/progenitor cells are able to proliferate in culture, are resistant to cryopreservation and have three interesting characteristics: plasticity, migration and engraftment.

- Embryonic stem cells and induced pluripotent stem cells

Pluripotent embryonic stem cells (ESC) are derived from the inner cell mass of blastocyst embryo. Several in vivo studies revealed the potency of ESC to differentiate into hepatocyte-like cells and to reduce induced liver fibrosis. Mouse ESC-derived green fluorescent protein (GFP)+ cells injected into CCl4-injured mice [81], undifferentiated mouse ESC injected into CCl4-treated mice [82] [83], and human differentiated ESC transplanted into CCl4-injured SCID mice [84] showed hepatic differentiation, integrated into liver parenchyma, and reduced liver fibrosis without evidence of tumorigenicity. The result of these studies should be further confirmed, however, since teratoma formations were observed in other studies. Splenic teratomas were formed in mice with

induced hepatocellular injury 35 days after administration of undifferentiated mouse ESC and 60 days after transplantation of mouse ESC-derived alpha-fetoprotein-producing cells [85]. Injection of undifferentiated mouse ESC into the spleen of immunosuppressed nude mice also gave rise to splenic teratomas [86]. Although ESC have the ability to differentiate into hepatocytes, their malignant potential and ethical issues still remain the major obstacles to develop ESC treatment in clinical settings. Moreover, there may be genetic/epigenetic changes and immune rejection problems when ESC are transplanted, due to their allogeneic nature [87].

To avoid these issues, new technologies have enabled tissue cells to become induced pluripotent stem cells (iPSC). Along with the development in the field of stem cell reprogramming, iPSC represent promising stem cells in cell-based liver therapy. Song and colleagues provided evidence of hepatocyte differentiation of human iPSC for the first time [88]. At various differentiation stages, human iPSC-derived hepatic cells from different organs repopulated the liver of mice with induced liver cirrhosis. The engraftment potential of differentiated iPSC was comparable to that of human hepatocytes and was higher than that of undifferentiated human ESC or iPCS [89]. iPSC provide an unlimited

source for regenerative medicine since patient-specific cells produce neither ethical issue nor problem of cell rejection. Despite iPSC's promise, potential risk of genetic manipulation and mutagenesis should be considered before any clinical application. Other issues remains to be addressed in recruiting iPSC are (1) the source of iPSC, whether patient-specific iPSC should be derived from diseased tissue portion, (2) directed hepatic differentiation protocol, and (3) extensive characterization of hepatic differentiation [90].

- Mesenchymal stem cells

Mesenchymal stem cells (MSCs) have extensively been investigated as potential therapeutic options for the treatment of various degenerative diseases and immune disorders, mainly because of their differentiation potential and immunoregulatory properties [91]. As compared to embryonic stem cells, MSCs do not give rise to ethical problems and have a safer profile in terms of oncogenicity [92].

The different MSC's properties make them an attractive therapeutic tool in the context of liver fibrosis, as it will be discussed in the following paragraphs.

CHAPTER 3

MSC's properties and their potential use in regenerative medicine

3.1. General features

In 2006, the International Society for Cellular Therapy proposed minimal criteria to define human MSCs [93]. First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, ≥ 95% of the MSC population must express CD105, CD73 and CD90, and lack the expression (≤ 2% positive) of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II surface molecules. Third, MSCs must differentiate into osteoblasts, adipocytes and chondroblasts under standard in vitro differentiating conditions [93].

MSCs are spindle-shaped fibroblast-like cells and have the ability of self-renewal. They can be isolated and expanded with high efficiency [94].

3.2. Differentiation potential

The high degree of plasticity of MSCs has widely been described during the last decade [95] [96] [97] [98].

MSCs have been shown to have the ability to differentiate into a variety of mesodermal cell lineages (including adipocytes, osteoblasts, chondroblasts, myocytes and cardiomyocytes) and into non-mesodermal cells (such as hepatocytes and neurons), depending on their microenvironment [99].

In particular, in vitro models provided evidence of the differentiation potential of MSCs into hepatocyte-like cells with functional properties such as albumin and urea production, glycogen storage, LDL uptake and phenobarbital-induced cytochrome p450 expression [100] [101].

Moreover, the in vivo hepatic differentiation of MSCs has been demonstrated in rats [102] [103], mice [104] and humans [105].

Adult-derived human liver stem/progenitor cells (ADHLSC) are obtained from healthy livers after a two-step collagenase perfusion and primary culture of the parenchymal fraction. The cells display a mesenchymal phenotype and a preferential hepatocyte expression

pattern [106] [107]. The cells cannot differentiate into other cell types (such as adipocytes and osteoblasts), like extra-hepatic MSCs. However, ADHLSC are able to differentiate into hepatocyte-like cells in vitro, after treatment with a cocktail of specific growth factors and cytokines. The differentiation was observed at the morphological, gene expression and functional levels. In vivo, once intrasplenically transplanted in immunodeficient mice, ADHLSC are able to migrate, engraft into the murine liver parenchyma and differentiate into hepatocyte-like cells up to 2 months post-transplantation. Recently, we demonstrated that engrafted ADHLSC are able to proliferate and participate to mouse liver regeneration after hepatectomy [108]. The cells have been successfully expanded in large scale and GMP conditions and transplanted in human trial [109]. The cells were approved as technological innovation by the European patents office in 2009 and as orphan drugs for the Crigler Najjar syndrome and urea cycle defects by the European Medicines agency. The technology has been successfully transferred to a spin-off company, Promethera Biosciences, for industrial and clinical developments.

This hepatic differentiation potential is essential for MSC-based therapies in the context of chronic liver diseases in which the injured hepatocytes are unable to regenerate [69].

3.3. Immunomodulatory properties

The ability of MSCs to modulate the immune response is attracting great interest, in the context of cell-based therapy and allogeneic transplantation.

It is well known that MSCs suppress the activity of cells from both adaptive and innate immunity. Indeed, MSCs can inhibit the proliferation of CD8+ cytotoxic lymphocytes and increase the relative proportion of CD4+ T helper-2 lymphocytes and CD4+ regulatory T lymphocytes [110] [111]. This effect on T lymphocytes indirectly suppresses the function of B lymphocytes as their activation is mainly T cell dependent. Moreover, MSCs can modulate B cells functions by inhibiting their proliferation, differentiation into antibody-secreting cells and chemotaxis. Soluble factors like transforming growth factor β1, hepatocyte growth factor, prostaglandin E2 and indoleamine 2,3-

dioxygenase seem to be implicated in this immunosuppressive activity [112].

MSCs also exert inhibitory effects on monocytes, dendritic cells, macrophages and NK cells, which belong to the innate immune system. MSCs inhibit the maturation of monocytes into dendritic cells, which play a role in antigen presentation to naïve T-cells. MSCs also inhibit the secretion of TNF-α, INF-ɤ and interleukin-12 by dendritic cells and increase their secretion of IL-10, reducing their proinflammatory potential [113] [114]. This inhibitory effect exerted by MSCs seems to be mediated by soluble factors, including prostaglandin E2 (PGE2) [115]. MSCs are also able to suppress NK cell's proliferation, cytolytic activity and secretion of cytokines. The role of PGE2 and indoleamine 2,3- dioxygenase has been established [116].

Thanks to all these characteristics, MSCs have generated a great interest for their potential use in regenerative medicine.

Altogether, while having the least potential to differentiate into endodermal cells compared to ESC and iPSC, MSCs can be readily obtained and expanded into large quantities. Moreover, MSCs are resistant to cryopreservation and maintain a stable phenotype

following the passages in culture [117]. Furthermore, the use of MSC sidesteps many obstacles for conducting human trials, such as ethical concerns, risk of rejection, and teratoma formation. Considering the unrelieved concerns regarding safety and efficacy, there has not been a clinical trial using human ESC and iPSC-derived hepatocytes for liver regeneration.

3.4. Homing and engraftment

MSCs have the potential to migrate to the injured site and thereafter to engraft into the concerned organ. This involves their ability to migrate across the endothelial cells and to integrate the organ.

It is well known that injured tissues express several receptors and ligands (such as CXCR4 and SDF-1) that facilitate the migration of MSCs to the damaged sites. Furthermore, chemokines are released following injury, creating a gradient followed by MSCs [118]. This represents a key mediator of trafficking of MSC to the site of injury. Finally, MSCs also express some integrins, selectins and chemokine receptors involved in the adhesion and migration of leucocytes [119] [120].

The advantage of this property is that MSCs can participate to liver regeneration and ensure continued delivery of trophic signal molecules. However, follow-up studies are necessary to assess the long-term engraftment rate of MSCs.

3.5. Therapeutic significance of MSC secretome

Soluble factors secreted by MSC have been described to play an important role in liver regeneration and to protect hepatocytes from cell death. It has been demonstrated that bone marrow MSC conditioned medium has an anti-apoptotic and pro-mitotic effect on cultured hepatocytes. Moreover, a systemic infusion of MSC conditioned medium could inhibit hepatocytes cell death and enhance liver regeneration in vivo, in a D-galactosamine-induced rat model of acute liver injury [121]. Zhang and colleagues demonstrated that human umbilical cord matrix stem cells provide a significant survival benefit in mice with CCl4-induced acute liver failure, through paracrine effects, by stimulating endogenous liver regeneration [122].

Beside liver regeneration, MSC secretome has also been described to have anti-fibrotic properties. Li and colleagues demonstrated that

transplantation of exosomes derived from human umbilical cord MSC could alleviate CCl4-induced liver fibrosis by inhibiting epithelial to mesenchymal transition (EMT) and by protecting hepatocytes [123].

CHAPTER 4

MSC-based therapy for liver fibrosis treatment: from in vitro studies to clinical trials

Over the past few years, an increasing number of studies have evaluated the anti-fibrotic potential of MSCs. In vivo studies highlighted the ability of MSCs to reduce liver fibrosis in animal models. In vitro studies aimed to elucidate the underlying mechanisms by which MSC could modulate HSC activation. Finally, clinical trials evaluate the efficiency of MSC transplantation for the treatment of liver fibrosis in humans.

4.1. Preclinical studies

Several in vivo studies were performed in order to evaluate the therapeutic potential of mesenchymal stem cells in the context of liver fibrosis (Table 2) [124] [125] [126] [127] [128] [129] [130] [131] [132].

Table 2: In vivo studies using MSCs to treat liver fibrosis

Species	Fibrosis induction	Administration route	MSC source	Number of cells injected/ animal	Results	Anti-fibrotic mechanisms proposed	Reference
Rats	CCl4 IP	Tail vein	Human umbilical cord blood	$1x10^6$	Liver fibrosis alleviated 4 weeks post-infusion Improvement of liver function	Differentiation into hepatocyte-like cells	[124]
Rats	CCl4 IP	Portal vein	Rat adipose tissue	$2x10^6$	Improvement of liver functional tests, histological findings and microcirculation 6 weeks post-infusion	Not mentioned	[125]
Mice	CCl4 IP	Intrahepatic	Murine bone marrow	$1x10^6$	Reduced fibrosis and apoptosis 30 days post-infusion Improvement of liver function	Not mentioned	[126]
Mice	CCl4 IP	Tail vein	Murine bone marrow	$1x10^6$	Thinner fibrotic areas and decreased collagen depositions 4 weeks post-infusion Improvement of liver function	Promotion of hepatocyte proliferation and modulation of inflammation	[127]
Rats	CCl4 SC	Portal vein	Human bone marrow	$1x10^6$	Reduced fibrosis 4 weeks post-infusion Improvement of liver function	Differentiation into hepatocyte-like cells Expression of MMPs by MSCs	[128]
Mice	CCl4 IP	Tail vein	Murine bone marrow	$1x10^6$	Decrease in liver fibrosis 4 weeks after transplantation	Increased expression of MMPs	[129]
Rats	CCl4 SC / DMN IP	Intraveinous	Rat bone marrow	$3x10^6$	Decrease in collagen deposition and of α-SMA expression Improvement of liver function	Not mentioned	[130]
Rats	CCl4 SC	Tail vein	Rat bone marrow	$3x10^6$	Decrease in collagen deposition Elevation of serum albumin	Not mentioned	[131]
Mice	CCl4 IP	Tail vein	Human bone marrow	$5x10^5$	Reduction in fibrosis 4 weeks after cell infusion	Enhanced expression of MMP-9 and decreased expression of α-SMA, TNFα and TGFβ	[132]

In most of the studies, liver fibrosis was induced by intraperitoneal or subcutaneous injection of CCl4. This model has the advantage to be the best characterized model with respect to histological, biochemical, cell and molecular changes associated with the development of liver fibrosis. Moreover, it can reproduce the pattern of most of the diseases seen in human fibrosis. However, this model has also some limitations as it is not a suitable model to study all kinds of liver fibrosis, such as biliary fibrosis. Moreover, it cannot provide a perfect simulation of a human disease as there are large species differences in immune reaction, gene expression/regulation, metabolic, pharmacological and tissue response [133].

The most studied MSCs are those from the bone marrow. These cells have been reported to be beneficial in the prevention of pulmonary fibrotic lesions [134]. However, the aspiration of the bone marrow remains an invasive procedure. The bleeding tendency of cirrhotic patients and their general condition may represent an obstacle for autologous cell transplantation.

Alternative sources of MSCs such as adipose tissue and umbilical blood cord have then been proposed but the number of studies in the context of liver fibrosis treatment remains limited, like studies using human MSCs in animal models. Most of the cell sources used in the in vivo studies are murine MSCs.

The results of the in vivo studies are promising as they report a decrease of the liver fibrosis with a frequent improvement of the hepatic functions. Most of the time, these results are observed 4 weeks after cell infusion. Long-term studies would be of great interest in order to evaluate if the observed anti-fibrotic effect persists over time. However, the CCl4 injections need to be continued after the MSC injection in order to avoid a regression of liver fibrosis. This represents an obstacle to the long-term studies, as animals can hardly support CCl4 injections during a long period of time. Beside an improvement of liver fibrosis and liver function, one study reported also an improvement in the liver microcirculation after MSC injection [125]. In two other studies, the decrease in the collagen deposition was correlated to a decrease of α-SMA expression, a classical marker of activated stellate cells [130] [132].

In vivo studies highlight the controversy that still remains about the exact mechanisms by which MSCs exert their beneficial effect. Indeed, some studies mention the differentiation of MSCs into hepatocyte-like cells [124] [128] and/ or the expression of metalloproteinases by MSCs [128] [129] [132]. The promotion of hepatocyte proliferation and the modulation of inflammation have also been proposed [127].

The question of the ideal route of MSCs administration remains one of the main unsolved issues regarding efficient injection of MSCs. Even if the tail vein seems to be the most often used administration route in animals, the portal vein [125] [128] and intrahepatic injections [126] also seem to be efficient. The optimal doses of cells also need to be evaluated as there are significant variations between studies in terms of number of cells injected per animal.

4.2. In vitro studies

As mentioned above, following liver injury, hepatic stellate cells (HSC) are activated into proliferative, α-smooth muscle actin positive, myofibroblast-like and extracellular matrix producing cells [14]. Hence, activated HSCs represent an attractive target for antifibrotic therapy.

Several in vitro studies demonstrated the ability of MSCs to modulate HSC's activation indirectly via paracrine mechanisms and directly through cell-cell contacts. The use of in vitro models is supported by the fact that HSC activation can be mimicked in vitro, when HSCs are in contact with the plastic culture dishes [14].

4.2.1. Paracrine mechanisms

Using indirect co-culture systems, Parekkadan and colleagues showed that human bone marrow-derived MSCs were able to inhibit the collagen synthesis in rat's activated HSC and, to a lesser extent, in immortalized human HSCs, as demonstrated by a significant reduction of procollagen type I C-Peptide secretion level. Moreover, MSCs could inhibit HSC's proliferation and induce their apoptosis, even if HSCs didn't revert to a quiescent state. The underlying mechanisms in the modulation of HSC activity by MSCs were attributed to IL-10, TNFα and HGF. IL-10 and TNFα secretion by MSCs seemed to inhibit synergistically the collagen secretion and the proliferation of HSCs while MSC-derived HGF induced apoptosis in activated HSCs, as demonstrated by antibody-neutralization studies [135].

Adipose tissue derived human MSCs could also indirectly inhibit murine HSC's proliferation. This growth inhibition is partially mediated by TGF-β3 and HGF, secreted by MSCs. Neutralization of both cytokines synergistically decreased the percentage of cells in the G0/G1 cell cycle phase. A decrease in the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 by MSCs seemed to be partially involved in the suppressive effect of MSCs on HSCs. Gene expression of collagen type I and III was also inhibited by MSCs [136].

NGF released from human bone marrow-derived MSCs may also represent an important paracrine loop by which human HSC's activation can be modulated. Using indirect co-culture systems, Lin and colleagues demonstrated that NGF could inhibit HSC's proliferation and promote their apoptosis. The same effect was reproduced using recombinant NGF. NF-κB and its target gene, Bcl-x_L, seem to take part in the regulation of this process [137].

4.2.2. Cell-cell contacts

Other studies evaluated the effects of direct interplay and juxtacrine signaling between MSCs and HSCs.

Rat bone marrow-derived MSCs were shown to significantly inhibit rat HSCs proliferation and to reduce their α-SMA expression level, through a cell-cell contact mode. The Notch pathway, known to induce cell cycle arrest, is activated following MSCs-HSCs contact. This signaling pathway may take part in the inhibition of HSCs proliferation. In addition, the PI3k/Akt pathway seems to be involved in HSC's growth inhibition by the Notch pathway [138].

Human bone marrow-derived MSCs were also shown to inhibit the proliferation and activation of HSCs (LX-2 cell line) through cell-cell contact and through the secretion of HGF. This HSC's modulation is mediated by an inhibition of the TLR4/NF-κB signaling pathway [139].

Taken together, these studies shed light on new insights regarding the mechanisms responsible for the anti-fibrotic effects of MSCs.

4.3. Clinical trials

Over the past few years, nine clinical trials using human MSCs to treat patient presenting liver fibrosis have been published (Table 3) [140] [141] [142] [143] [144] [145] [146] [147] [148].

Table 3: Clinical trials using MSC to treat liver fibrosis

Cell source	Administration route	Number of cells infused	Patient population	Number of patients	Follow up period	Endpoints	Efficacy	Reference
Umbilical cord	Intraveinous	5 x 10^5 / kg , 3 times	Chronic hepatitis B	30 treatment 15 control	1 year	Safety/ Efficacy	Improvement of liver function and MELD score Reduced acites	[140]
Umbilical cord	Intraveinous	5 x 10^5 / kg , 3 times	Chronic hepatitis B	24 treatment 19 control	48 or 72 weeks	Safety/ Efficacy	Improvement of liver function and MELD score Increased survival rates	[141]
Umbilical cord	Intraveinous	5 x 10^5 / kg, 3 times	Primary biliary cirrhosis	7	48 weeks	Safety/ Efficacy	Decrease in serum alkaline phosphatase and ɣ-glutamyltransferase levels Alleviation of fatigue and pruritus Decrease of ascites	[142]
Bone marrow	Intraveinous	30 x 10^6/ patient	3 cryptogenic 1 autoimmune hepatitis	4	1 year	Safety/ Efficacy	Improvement of MELD score	[143]
Bone marrow	Intraveinous (peripheral vein or portal vein)	30-50 x 10^6/ patient	4 Chronic hepatitis B 1 Chronic hepatitis C 1 alcoholic cirrhosis 2 cryptogenic	8	24 weeks	Safety/ Efficacy	Improvement of liver function and MELD score	[144]
Bone marrow	Hepatic artery	3,4 x 10^8/ patient	Chronic hepatitis B	53 treatment 105 control	192 weeks	Safety/ Efficacy	Improvement of Alb, TBIL, PT and MELD score	[145]
Bone marrow (undifferentiated and differentiated MSC)	Intraveinous	1 x 10^6 / kg	Chronic hepatitis C	15 treatment 10 control	6 months	Efficacy	Improvement of liver function and MELD score	[146]
Bone marrow	Intrasplenic	10 x 10^6/ patient	Chronic hepatitis C	20	6 months	Safety/ Efficacy	Decrease od TBIL, AST, ALT, PT and INR Increase of the albumin levels	[147]
Bone marrow	Hepatic artery	5 x 10^7/ patient, twice	Alcoholic cirrhosis	12	12 weeks	Efficacy	Histological improvements Improvement of Child-Pugh score Decrease of TGF-β1, Collagen type 1 and α-SMA	[148]

The endpoints of the studies were to evaluate the safety and efficacy of bone marrow and umbilical cord MSCs transplantation. The cells were mostly infused intravenously even if two studies report infusions via the hepatic artery [145] [148] and in one study, the cells were even injected into the spleen [147]. There is a great variation in the number of cells infused per patient and in the frequency of injection in the different trials. The results of the studies seem promising in terms of improvement of liver function and MELD score. However, there is a lack in the evaluation of the liver histology after cell transplantation, except in one study reporting histological improvements [148].

Globally, the size of the samples is small in most studies and there is a lack of controls in five studies. The follow up period is quite short, except in one study with 192 weeks follow up. We think that it is crucial to evaluate the long term efficacy, prognosis and safety before proposing this therapy routinely in the clinical practice.

Using other types of MSCs and other patient populations could also be of great interest, in order to evaluate the best therapeutic option for each pathology.

The use of MSCs in the clinical practice is currently hindered by the incapacity to monitor the transplanted cells in the patients and by the lack of standardized transplantation protocols. Standardized protocols providing informations concerning the timing of cell injection following the stage of liver fibrosis, the number of cells and the administration route would be useful.

Only randomized controlled clinical trials are able to assess the potential clinical benefit of MSCs for patients affected by liver fibrosis. According to the clinical trials Website of the United States sponsored by the National Institutes of Health (http://clinicaltrials.gov), approximately 24 clinical trials are currently ongoing.

CHAPTER 5

Future prospects

MSCs may represent a clinical relevant solution for the treatment of liver fibrosis, given their interesting properties and the promising results of the preclinical and clinical studies.

However, several issues need to be clarified before MSC can be routinely proposed as a therapeutic option to treat liver fibrosis.

Over the past few years, concerns have been raised about the long-term effectiveness of MSCs cell-based therapy and the potential tumorigenic risk. Several evidences suggest that MSCs might promote tumor growth in vivo [149] [150] [151]. On the other hand, thanks to their immunomodulatory properties, MSCs may have an antitumor effect, in relation with the modulation of the inflammatory environment that characterizes many tumors [152] [153] [154]. MSCs are also able to interact with cancer cells and to inhibit signaling pathways associated with tumor growth and cell division [155] [156].

Moreover, there is a lack of standardized protocols for MSC transplantation. The optimal MSC doses, the timing and frequency of

injection and the administration route differ considerably between the different studies.

For all these reasons, we think that further studies, and in particular randomized controlled trials, are needed in order to evaluate the long term safety and efficacy of MSC-based treatment. Moreover, potency tests performed on MSCs before the injection to patients could be useful.

Conclusion

Although considerable advances have been made in the past decade to better understand the cellular and molecular mechanisms underlying liver fibrogenesis, no efficient therapy is available so far to treat this serious condition.

Further investigations and efforts are currently conducted to efficiently reverse liver fibrosis. MSC-based therapy has been shown to have a significant potential to decrease mortality and to improve the quality of life of patients suffering from liver fibrosis. A standardization is however needed before proposing this strategy routinely in clinical practice.

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Part II

Thesis objectives

As described in the introduction, liver fibrosis is a worldwide health problem. Except orthotopic liver transplantation, no effective treatment is available so far. Organ shortage being an increasing daily reality, there is an urgent need to find alternative therapeutic strategies.

The most promising emerging strategy seems to be cell based therapy and in particular mesenchymal stem cells.

Adult-Derived Human Liver Stem/progenitor Cells (ADHLSCs) share similar characteristics with extra-hepatic MSCs but are more closely related to the liver.

The objective of the current work is to evaluate the anti-fibrotic properties of ADHLSCs by studying their interactions with human hepatic stellate cells (HSCs), the main cell type implicated in the extracellular matrix production.

To address this issue, our first aim was to find tools that could allow the discrimination between ADHLSCs and HSCs.

Thereafter, our second aim was to evaluate the ability of ADHLSCs to modulate HSC activation process in vitro.

Part III

Results

Paper 1

Gene expression profiling and secretome analysis differentiate Adult-Derived Human Liver Stem/progenitor Cells and human hepatic stellate cells.

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The first part of this thesis work focuses on an in-depth comparative analysis of both Adult-Derived Human Liver Stem/progenitor Cells (ADHLSCs) and human hepatic stellate cells (HSCs). Indeed, the two cell types that were investigated in the current study are derived from the adult human liver and present numerous similar characteristics, including their morphology. The aim of this study is to assess the tools that may help to accurately discriminate both cell types and confirm their singularity before analyzing the study of their interactions.

In this work, we demonstrated that the studied cell populations present distinct gene expression and secretome profiles. Of importance, we also noticed that ADHLSCs secrete molecules of therapeutic and immunomodulatory importance that may support the anti-fibrotic effect of ADHLSCs.

Abstract

Adult-derived human liver stem/progenitor cells (ADHLSC) are obtained after primary culture of the liver parenchymal fraction. The cells are of fibroblastic morphology and exhibit a hepato-mesenchymal phenotype. Hepatic stellate cells (HSC) derived from the liver non-parenchymal fraction, present a comparable morphology as ADHLSC. Because both ADHLSC and HSC are described as liver stem/progenitor cells, we strived to extensively compare both cell populations at different levels and to propose tools demonstrating their singularity.

ADHLSC and HSC were isolated from the liver of four different donors, expanded in vitro and followed from passage 5 until passage 11. Cell characterization was performed using immunocytochemistry, western blotting, flow cytometry, and gene microarray analyses. The secretion profile of the cells was evaluated using Elisa and multiplex Luminex assays.

Both cell types expressed α-smooth muscle actin, vimentin, fibronectin, CD73 and CD90 in accordance with their mesenchymal origin. Microarray analysis revealed significant differences in gene expression profiles. HSC present high expression levels of neuronal markers as well

as cytokeratins. Such differences were confirmed using immunocytochemistry and western blotting assays. Furthermore, both cell types displayed distinct secretion profiles as ADHLSC highly secreted cytokines of therapeutic and immuno-modulatory importance, like HGF, interferon-ɤ and IL-10.

Our study demonstrates that ADHLSC and HSC are distinct liver fibroblastic cell populations exhibiting significant different expression and secretion profiles.

Introduction

The liver is composed of parenchymal and non-parenchymal cell populations. Complex and well-organized interactions between such cell types allow a perfect coordination of the liver functions for preservation of the systemic homeostasis. Indeed, the liver is concomitantly managing numerous important functions such as metabolism, protein synthesis and detoxification. Hepatocytes are the main parenchymal cell type and represent the most important functional one. Liver non-parenchymal cells include epithelial bile duct cells, non-epithelial Kupffer cells, sinusoidal endothelial cells and hepatic stellate cells (HSCs) [1].

Spindle shaped HSCs are located in the space of Disse between hepatocytes and sinusoidal endothelial cells [2]. The HSC population represents about 15% of the total number of resident cells in the normal liver. These cells have several important functions including retinyl ester storage and homeostasis, remodeling of extracellular matrix, production of growth factors and cytokines, contraction and dilatation of the sinusoidal lumen [3]. During liver injury, HSC are "activated" and evolve to myofibroblast-like cells. This activation is

characterized by an increase in cell proliferation and extracellular matrix protein deposition. At the structural level, activated HSC lose their big Vitamin A-containing lipid droplets and up-regulate the expression of some cell adhesion molecules like ICAM-1, VCAM-1 and NCAM and of α-smooth muscle actin as well as the secretion of pro-inflammatory cytokines [4] [5]. In vitro, part of this activation process is mimicked by culturing the cells on plastic culture dishes [6].

Our group previously obtained stem/progenitor cells from healthy adult human liver (ADHLSC). These expandable cells present a hepato-mesenchymal phenotype and have the potential to differentiate into hepatocyte-like cells both in vitro and in vivo [7] [8] [9]. Cultured ADHLSC exhibit a striking phenotypical resemblance with culture activated HSCs. Moreover, alike ADHLSCs, quiescent HSCs have been reported to express molecular markers of stem/progenitor cells and to be involved in liver regeneration [7] [10] [11].

In the current study, we carried out an extensive comparison between HSCs and ADHLSCs in order to assess the unique identity of ADHLSCs and to identify tools that can be used to differentiate both populations. To this end, we compared these mesenchymal cells after isolation from

the same liver by following their phenotype, genotype and behavior in vitro from passage 5 until passage 11.

We report several characteristics similar to both cell types but shed light on significant gene expression profile and functional differences. This study confirms the unique characteristics of ADHLSCs and demonstrates their secretion potential of cytokines that could be of therapeutic and immuno-modulatory importance.

Materials and methods

ADHLSC and HSC isolation and culture

The protocol and experiments were approved by the ethical committees of the St-Luc Hospital and faculty of Medicine of Université Catholique de Louvain. An agreement from the Belgian Ministry of Health was obtained for the Hepatocytes and Hepatic Stem Cells Bank. A written and signed informed consent has been obtained for each human liver used in the current study.

Four donors were used in the current study (Table 1).

Table 1: Characteristics of the four liver donors from which HSC and ADHLSC were isolated

Donor number	Age	Gender	Reason of death	Blood group	Ischemia time
89	3 days	M	Respiratory	A+	4 hours
93	2 years	F	Metabolic disease (liver transplanted)	O+	1 hour 43
97	7 months	F	Meningitis	A+	5 hours 30
98	7 days	M	Cardio-respiratory arrest	O-	4 hours 20

ADHLSC were obtained subsequently to primary culture of the liver parenchymal fraction previously obtained after a two-step collagenase perfusion, filtration and low speed centrifugation [7]. HSCs were isolated from the corresponding non-parenchymal fraction using a Nycodenz gradient centrifugation step (Myegaard, Oslo, Norway) [12].

Both cell types were cultured using DMEM containing 4.5g/L glucose (Invitrogen) supplemented with 10% Fetal Calf Serum (PAA) and 1% Penicillin/Streptomycin (Invitrogen), at 37°C in a fully humidified atmosphere (5% CO2). When reaching 80% confluence, cells were lifted with 0.05% trypsin-EDTA (Invitrogen) and replated at a density of

5000 cells/cm^2. The viability of recovered cells was evaluated using trypan blue exclusion assay.

Immuno-cytochemistry

Cells were fixed using paraformaldehyde 3.5%, for 15 min at room temperature. Endogenous peroxidase was eliminated using hydrogen peroxide 3.3% for 3 minutes. All steps were performed at room temperature. Cells were permeabilized using D-PBS containing 1% Triton X-100 (Sigma) for 10 minutes. Non-specific immuno-staining was prevented by 1h incubation in D-PBS containing 1% Bovine Serum Albumin (Sigma). Thereafter, cells were incubated with primary antibody for 1h (Table 2).

Table 2: Primary antibodies used for the phenotypic characterization of ADHLSC and HSC by immunocytochemistry & western blotting

Antibody	Supplier	Reference	Concentration used
ASMA	Dako	M0851	1/100
CK-18	Dako	M7010	1/350
CK-19	Dako	M0888	1/350
Desmin	Abcam	Ab6322	1/50
Fibronectin	Abcam	Ab32419	1/100
NCAM	Abcam	Ab9018	1/100
Nestin	Abcam	Ab22035	1/1000
Vimentin	Progen	105-15	1/100

After washing, cells were incubated with secondary antibody (EnVision – Dako) during 30 minutes. Detection was performed after 5 minutes incubation with liquid DAB and substrate chromogen (Dako). Counterstaining was performed using Mayer's hematoxylin for 10 minutes. Preparations were then mounted for microscopic analysis (DMIL, Leica, Belgium).

Western Blotting

Extracted Protein concentrations were determined using the BCA protein assay kit (Thermo Scientific). Fifty micrograms of protein samples were boiled for 10 min at 100°C and prepared for loading by adding dithiotreitol and bromophenol blue. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes. Blots were blocked with 5% milk powder in TBS with 0,2% tween (TBS-T). Following a washing step with TBS-T, membranes were incubated overnight with following primary antibodies and dilutions: CK18 (1:350 [DAKO]), CK19 (1:350 [DAKO]). Antibodies were diluted in blocking buffer. After additional TBS-T washes, membranes were incubated with secondary antibody for 1h (dilution: 1/20.000). The antigens were detected by enhanced chemiluminescence using ECL substrate.

Gene expression profile analysis

RNA was quantified with a Nanodrop1000 (Thermo Scientific) and RNA integrity evaluated with a Bioanalyzer 2100 (Agilent Technologies). 500pg-50ng total RNA were amplified with an Ovation Pico SL V2 Kit (NuGENE) and labelled with an Encore Biotin Module (NuGENE)

following standard procedures. HG-U219 plates were hybridized with an automated array processing GeneTitan (Affymetrix).

Affymetrix gene expression data were normalized using the robust multi-array algorithm [13] using a custom probe set definition that mapped probes to 18567 Entrez Gene Ids (HGU219_Hs_ENTREZG) [14]. Genes with a coefficient of variation lower than 0.03 were eliminated, resulting in a set of 13925 genes.

For the detection of differentially expressed genes, a linear model was fitted to the data and empirical Bayes moderated statistics were calculated using the limma package from Bioconductor [15]. Adjustment of p-values was done by the determination of false discovery rates (FDR) using the Benjamini-Hochberg procedure [15]. Genes representing a change of 1.5-fold or greater and moderated p-value <0.05 were considered as differentially expressed.

Functional analysis of gene expression data was conducted using the R/Bioconductor package GOstats and the GO database (http://www.geneontology.org). Only genes that could be associated with a unique Entrez Gene ID were used. Among those, only the genes representing a fold change of 1.5 or greater and a moderated p-value<0,05 were selected. The hypergeometric distribution was used to

evaluate the probability of randomly observing the enrichment for each GO term [16]. The data are deposited at Gene Expression Omnibus under reference number GSE49995.

Gene Set Enrichment Analysis (GSEA) was employed to identify biological pathways significantly associated with the ADHLSC. In comparison to other strategies for analysis of molecular profiling data that focus on high scoring individual genes, GSEA does not employ a significance threshold and evaluates microarray data at the level of gene sets defined based on prior biological knowledge. This approach has been reported to yield robust results even when dealing with heterogeneous samples with subtle sample class differences. For the current analysis, gene sets were extracted from the full Molecular Signature Database (MSigDB v.4-0). Additionally the subset of canonical pathway is analyzed alone. Analyses were based on a Signal2noise metric and a weighted scoring scheme with 1000 permutations on gene sets. Only gene sets with more than 15 genes were included in the analysis.

RT-qPCR

Total RNA was extracted from 1.5 million cells for each cell population using Tripure isolation reagent (Roche). cDNA was generated from 1µg of RNA using the Thermoscript™ RT kit (Invitrogen) according to manufacturer's instructions. qPCR was carried out in duplicate using TaqMan Gene expression Master Mix (Applied Biosystems) and pre-designed TaqMan probes and primers obtained from Applied Biosystems. The amplification was performed using the StepOnePlus Real-time PCR machine. The housekeeping gene cyclophilin A (PPIA) was used as a reference gene for normalization and water was used as a negative control.

Flow cytometry

Cells were re-suspended in D-PBS at a concentration of $2x10^5$ cells /ml. For intracellular immunostaining, cell permeabilization was performed with cytofix/cytoperm for 20 minutes at 4°C (BD Pharmingen). Cells were then washed and incubated for 30 minutes at 4°C with the antibodies (see Table 3).

Table 3: Antibodies used for the phenotypic characterization of ADHLSCs and HSCs by flow cytometry

Antibody	Fluorochrome	Corresponding isotype	Supplier	Reference	Concentration
Anti-ASMA	FITC	MsIgG2a	Abcam	Ab8211	1/10
Anti-CD29	APC	MsIgG1, k	BD	559883	1/10
Anti-CD44	FITC	MsIgG2b, k	BD	555478	1/10
Anti-CD45	PE-Cy7	MsIgG1, k	BD	557748	1/10
Anti-CD73	PE	MsIgG1, k	BD	550257	1/10
Anti-CD90	APC	MsIgG1, k	BD	559869	1/10
Anti-CD117	APC	MsIgG1, k	BD	550412	1/10
Anti-CD133/2	PE	MsIgG1	Miltenyi	130-080-901	1/5

The corresponding control isotypes were used to evaluate the non-specific binding. After washing, cells were suspended in Stabilizing Fixative (BD Pharmingen) before reading with a CANTO II flow cytometer. The analyses were performed using the BD FACSDiva Software.

ELISA

When 60-70% of confluence was reached, the cells were washed and the conditioned medium was replaced by a serum-free medium. After

24 hours incubation, supernatants were collected and cells were lifted for counting and viability evaluation using trypan blue exclusion assay.

For the collagen secretion analysis, we used an Elisa kit for the procollagen type I C-Peptide (Takara Bio Inc, Japan). Hepatocyte Growth Factor (HGF) and Transforming Growth Factor beta 1 (TGFβ1) levels in the culture supernatants were assayed by using Quantikine Elisa Kits from R&D Systems. The experiments were performed according to the manufacturer's instructions. For TGFβ1 Elisa, samples were activated by acidification followed by neutralization in order to activate latent TGFβ1 to immune-reactive TGFβ1 detectable by the Quantikine TGFβ1 immunoassay. The measurement of the absorbance at 450 nm was done with a Victor X4 plate Reader (PerkinElmer). For HGF and TGFβ1 kits, a reading at 570 nm was subtracted to the 450 nm reading to correct the optical imperfections of the plates.

Luminex analysis

We also used a 27plex kit (IL-1b, IP-10, IL-2, IL-4, IL-6, IL-7, IL-9, IL-10, IL-13, IL-15, Eotaxin, FGF, GM-CSF, INF-ɤ, MIP-1a, MIP-1b, RANTES, TNFα, IL-1ra, IL-5, IL-8, IL-12, IL-17, G-CSF, MCP-1, PDGF-bb and VEGF) and the Luminex technology (Bio-Plex 200, Biorad) to investigate the

secretome of both liver cell types . The principle of the technique is based on color-coded beads and enables to detect up to 100 cytokines simultaneously. The primary antibody directed against the target protein is conjugated with the dyed beads. After several washes to remove unbounded proteins, a secondary biotinylated antibody is added to the reaction. Streptavidin-phycoerythrin (Streptavidin-PE) is then added to bind the biotinylated antibody. By measuring the relative fluorescence intensity, the antigen-antibody reaction can be measured. The assays were performed following the manufacturer's instructions.

Briefly, after the pre-wetting of the plate, 50µl of the beads were added in each well and washed twice. 50 µl of the samples (serum free culture supernatants recovered after 24 hours of culture) were added to the plate. The plate was shaken during 30 seconds and then incubated for 45 minutes on a plate shaker at 120 rpm at room temperature. The plate was washed three times with the Bio-Plex wash buffer and 25µl of the detection antibody was added in each well and incubated for 30 minutes on a plate shaker at 120 rpm. The plate was then washed three times with the Bio-Plex wash buffer and 50 µl of the Streptavidin-PE solution was added in each well. The plate was shaken during 30 seconds and incubated for 10 minutes on a plate shaker at 120 rpm.

Finally, after three washes of the plate with the Bio-Plex wash buffer, the beads were resuspended with 125µl of Bio-Plex Assay Buffer. The plate was read by the Luminex machine and the data were analyzed using Bio-Plex Manager 6.0.

Statistics

Results, others than those of microarray studies, are expressed as mean ± standard error of the mean (SEM). Statistical differences were determined by Student's *t* test for two groups' comparison. Differences were considered significant when p values $*p<0.05$, $**p<0.01$, $***p<0.001$.

Results

Phenotypic and genotypic characterization of ADHLSC and HSC

For each liver donor, HSC and ADHLSC were cultivated under the same culture conditions and concomitantly followed. The fibroblastic morphology displayed by both cell types remained stable over the different studied passages (Figure 1A). The population cumulative doubling was similar for the two cell types (Figure 1B).

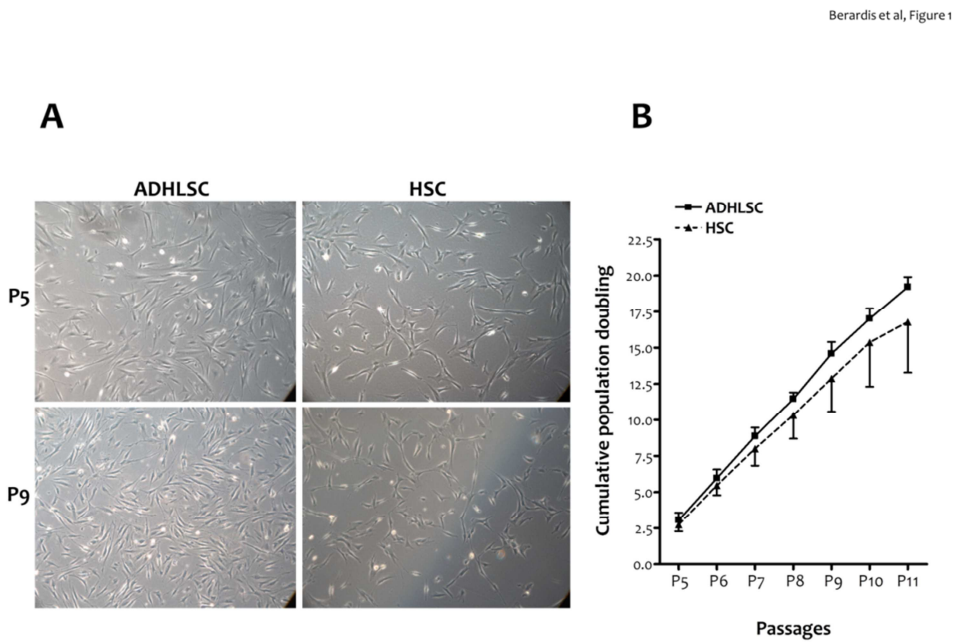


Figure 1. ADHLSC and HSC in culture. *A*, Fibroblastic morphology at passages 5 & 9 as shown using phase contrast microscopy, original magnification 100x. *B*, Cumulative population doubling of both cell types from passage 5 to passage 11 (n=4).

We investigated the mesenchymal phenotype of both cell types (n=4) by exploring the expression of several specific appropriate markers using flow cytometry. Both cell types were immuno-positive for most of the membrane markers widely used to characterize mesenchymal stem cells. This was the case for CD73, CD90, CD29, and CD44 for which expression levels remained stable for the analyzed passages (Table 4).

Table 4 : Phenotypic characterization of ADHLSC and HSC by flow cytometry for mesenchymal stem cells, hematopoietic cells and extracellular matrix markers. Data presented as mean percentage of immunopositive cells from four different donors at early (P5) and late(P11) passages. No significant difference was observed between the two cell populations.

		ADHLSC		HSC	
		P5	P11	P5	P11
Mesenchymal stem cells markers					
	ASMA	87,8 ± 4,7%	83,8 ± 2,9%	91,9 ± 5,5%	87,4 ± 12,6%
	CD73	88,2 ± 5,9%	90,2 ± 6,1%	89,9 ± 2,5%	82,8 ± 20,1%
	CD90	97,2 ± 2,7%	92,7 ± 7,8%	95,6 ± 2,3%	88,7 ± 10,4%
Hematopoietic cells markers					
	CD133	0,7 ± 0,2%	1,3 ± 0,3%	2,1 ± 2,2%	2,2 ± 2,3%
	CD45	1,4 ± 0,8%	2,6 ± 3,1%	1,7 ± 1,0%	1,8 ± 1,9%
	CD117	0 ± 0%	0,07 ± 0,12%	0,3 ± 0,4%	0,03 ± 0,06%
Extracellular matrix markers					
	CD49b	95,6 ± 1,1%	95,5 ± 2%	89,7 ± 8,0%	90 ± 9,7%
	CD29	86,4 ± 10,2%	60,8 ± 11%	77 ± 21,5%	56,6 ± 49,64%
	CD44	77,5 ± 8,6%	79,7 ± 9,2%	68 ± 4,1%	75 ± 18,9%

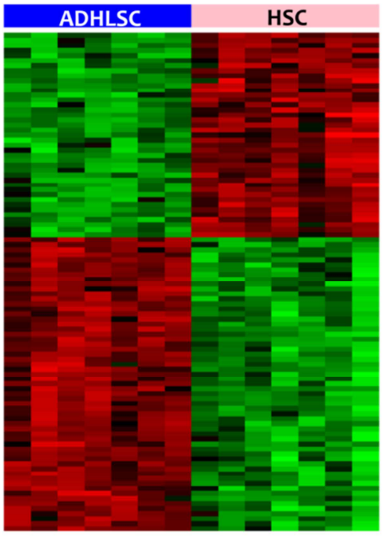
No significant difference was noticed between HSC and ADHLSC. These findings were confirmed using immunocytochemistry and RT-qPCR after analyzing the expression of other classical mesenchymal markers like vimentin, α-smooth muscle actin and fibronectin (data not shown). The mesenchymal phenotype of both cell types was also supported by the negative expression of hematopoietic markers like CD45, CD117 and CD133 as demonstrated using flow cytometry (Table 4).

This first comparative characterization only confirmed the mesenchymal phenotype of ADHLSCs and HSCs but did not allow us to discriminate both cell populations. We thereafter studied the gene expression profile of ADHLSCs and HSCs using Affymetrix HG-U219 gene chips expression. The analysis was performed on 7 different samples for each cell population (4 samples from the four donors at Passage 5 + 3 samples from only 3 donors at Passage 7). These experiments allowed us to point out significant signature differences (100 top genes that are significantly differentially expressed in the two cell populations) after screening of 13925 genes (Figure 2A). We noticed that liver stem/progenitor cells highly and predominantly expressed chemokine ligands such as CXCL1, CXCL3, CXCL5, CXCL6 and CXCL7 when compared to HSCs (Figure 2B). ADHLSCs also displayed

increased levels of cytokines like IL-1β, IL-6, IL-8, IL-33, and LIF as well as of growth factors like HGF in comparison with HSC (Figure 2B).

Berardis et al, Figure 2

A



B

Gene Symbol	Gene Name	Ratio HSC/ADHLSC	p-Value
CCL2	*chemokine (C-C motif) ligand 2*	-1,769	0,006
CCL20	*chemokine (C-C motif) ligand 20*	-2,674	0,016
CCL5	*chemokine (C-C motif) ligand 5*	-1,551	0,012
CCL7	*chemokine (C-C motif) ligand 7*	-3,889	0,015
CXCL1	*chemokine (C-X-C motif) ligand 1*	-11,316	0,001
CXCL2	*chemokine (C-X-C motif) ligand 2*	-2,817	0,036
CXCL3	*chemokine (C-X-C motif) ligand 3*	-3,565	0,003
CXCL5	*chemokine (C-X-C motif) ligand 5*	-7,153	0,004
CXCL6	*chemokine (C-X-C motif) ligand 6*	-12,023	0,007
IL1B	*interleukin 1, beta*	-8,424	0,000
IL6	*interleukin 6*	-1,672	0,003
IL8	*interleukin 8*	-7,737	0,001
IL33	*interleukin 33*	-4,386	0,007
LIF	*leukemia inhibitory factor*	-3,178	0,000
HGF	*hepatocyte growth factor*	-3,532	0,025

Figure 2. Gene expression profile analysis of ADHLSC and HSC using microarrays. *A*, Hundred top genes significantly and differentially expressed in the two cell populations (4 samples from passage 5 for all donors and 3 samples at passage 7 for 3 donors including the diseased donor). Red bars denote over-expression, black bars denote equally expression and green bars denote under-expression. B, chemokines and cytokines expression profile.

Regarding cytoplasmic markers, HSCs expressed significantly higher amounts of desmin (type III intermediate filament), elastin,

desmoplakin (anchors intermediate filaments to desmosomal plaques) and dystrophin as compared to ADHLSC (Figure 2C).

When comparing MMP expression between the two cell types, we noticed an elevated expression of MMP2, MMP3 and MMP14 in ADHLSCs and an increased expression of MMP16 in HSCs (Figure 2C).

C

Gene Symbol	Gene Name	Ratio HSC/ADHLSC	p-Value
DES	*desmin*	1,691	0,032
DSP	*desmoplakin*	7,916	0,000
ELN	*elastin*	5,544	0,000
DMD	*dystrophin*	2,369	0,000
KRT14	*keratin 14*	4,104	0,009
KRT18	*keratin 18*	2,934	0,004
KRT19	*keratin 19*	4,508	0,001
KRT34	*keratin 34*	3,721	0,011
KRT7	*keratin 7*	3,389	0,010
MMP14	*matrix metallopeptidase 14*	-1,504	0,003
MMP16	*matrix metallopeptidase 16*	1,716	0,017
MMP2	*matrix metallopeptidase 2*	-1,761	0,008
MMP3	*matrix metallopeptidase 3*	-5,359	0,014
NGF	*nerve growth factor (beta polypeptide)*	1,820	0,021
NEDD1	*neural precursor cell expressed, developmentally down-regulated 1*	1,510	0,002
NEDD9	*neural precursor cell expressed, developmentally down-regulated 9*	1,984	0,002
NDNF	*neuron-derived neurotrophic factor*	2,102	0,006
ANK2	*ankyrin 2, neuronal*	1,689	0,020

Figure 2. Gene expression profile analysis of ADHLSC and HSC using microarrays.

C, intracytoplasmic, intermediate filaments, and neural growth factors.

In order to obtain a functional annotation of our gene expression results, we used the Gene Set Enrichment Analysis (GSEA) method. We

have used GSEA to identify those most relevant “canonical” pathways and Gene ontology categories enriched in ADHLSC. Results are included in Figure 2D and Table 5.

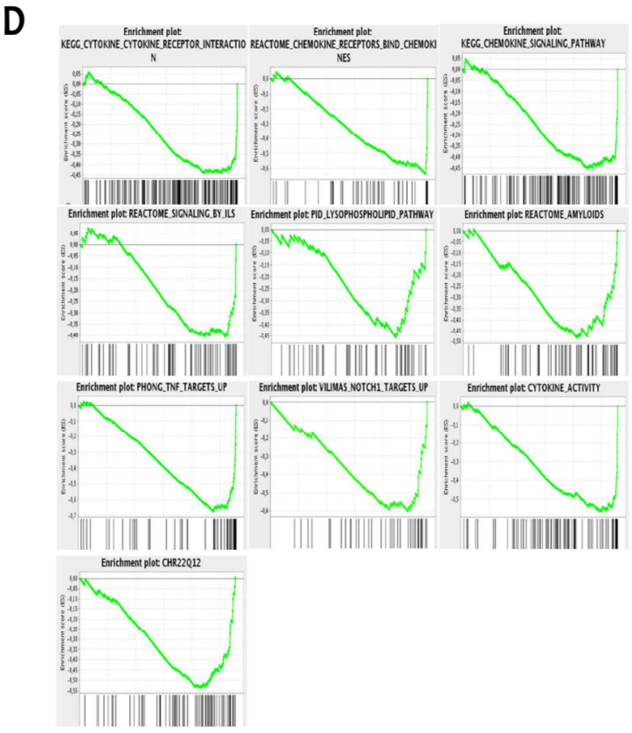


Figure 2. **Gene expression profile analysis of ADHLSC and HSC using microarrays.** D, Gene Set Enrichment Analysis (GSEA). Selected statistically significant GSEA plots obtained from enriched ADHLSC. Within these plots, the green line represents the sliding enrichment score and the black bars demarcate the position of the gene set members within the ranked expression data.

Table 5 : Gene Set Enrichment Analysis biological: Pathways significantly associated with ADHLSC and the corresponding FDR as well as the enriched genes

GENESET	SIZE	FDR_P VALUE	ENRICHED GENES
KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	213	0,000	CXCL5 CXCL1 CSF3 IL1B CXCL3 CXCL6 BMP2 IL8 CCL7 HGF CSF2 IL11 CCL20 LIF CCL11 TGFB1 TNFRSF19 CXCL2 IL24 IL1A CCL5 TNFRSF21 CCL8 CLCF1 EPOR CRLF2 IL19 IL15RA CCL24 TNFRSF10A RELT CNTFR FLT3 TNFRSF12A TGFB2 IL17RA CSF1 TNFRSF1A VEGFA CXCR3 AMHR2 AMH TNFRSF10B CCL23 CCL2 TGFBR2 TNFRSF1B CCL26 IL6 CCL3 IL7R CCR9 LTB IL18R1 TNFSF15 IL17RB EPO NGFR CCL4 CD40 TNFSF4 CXCL10 PDGFB IL10RB TNFSF8 CX3CL1 ACVR1B CD27 IL2RG TNFRSF8 FLT4 IFNE TNFSF14 TNFRSF10C CCR7
REACTOME_CHEMOKINE_RECEPTORS_BIND_CHEMOKINES	46	0,000	CXCL5 CXCL1 CXCL3 CXCL6 IL8 CCL7 CCL20 CCL11 CXCL2 CCL5
KEGG_CHEMOKINE_SIGNALING_PATHWAY	135	0,002	CXCL5 CXCL1 CXCL3 CXCL6 IL8 CCL7 CCL20 GNB2 CCL11 RAC2 CXCL2 CCL5 CCL8 PRKX PIK3CD

			TIAM2 SHC1 CCL24 GNAI2 PIK3R2 PREX1 CXCR3 BCAR1 NFKBIA CCL23 CCL2 CCL26 GNG2 NFKBIB CCL3 CCR9 PRKACA IKBKG CSK ADRBK1 CCL4 PXN PRKCZ CXCL10 STAT2 CX3CL1
REACTOME_SIGNALING_BY_ILS	70	0,041	IL1B IRAK3 HGF CSF2 IL1A PIK3CD SOCS3 SHC1 NFKB2 IRAK2 TAB1 PIK3R2 CASP1 GAB2 PELI3 IL6 IL7R IKBKG MYD88 IRAK1 SQSTM1 PELI2 MAP2K4 NOD2 IL2RG
PID_LYSOPHOSPHOLIPID_PATHWAY	51	0,028	IL8 GNA11 TRIP6 GNAI2 MMP2 ARHGEF1 BCAR1 NFKBIA PLCG1 GNG2 IL6 HBEGF FOS GNAZ JUN ADRA1B PRKCE PXN GNA12 PLD2 ADCY5
REACTOME_AMYLOIDS	59	0,009	HIST1H3I HIST1H2BM HIST1H2BN CST3 HIST1H4K HIST4H4 HIST1H3F HIST1H2BL SNCA HIST1H3B HIST1H2BF HIST1H3A HIST3H2BB HIST1H2BH HIST1H2BG HIST2H2BE HIST1H4L HIST1H3D HIST1H2BC HIST1H4D HIST1H3E HIST1H3G HIST1H3J HIST1H2BE HIST1H2BO HIST1H2AJ HIST1H2BI HIST1H4B HIST1H4H MFGE8
VILIMAS_NOTCH1_TARGETS_UP	40	0,001	BCL2A1 ICAM1 CCL5 HEY1 JUNB EGR1 NFKB2 THY1 EGR2 RELB BIRC3 DTX1 CARD11 NFKBIA ZAP70 P2RY10
PHONG_TNF_TARGETS_UP	56	0,000	CXCL1 CXCL3 BMP2 IL8 CSF2 IL11 CCL20 LIF ICAM1 CXCL2 ETS2 PLAU IER2 LDLR TNFAIP3 IRF1 BTG1 JUNB EGR1 NFKB2 NKX3-1 ATF3 EGR2 BIRC3 DUSP1 NFKBIA

			TNFRSF10B REL IL6 FOS SDC4 JUN MCL1
CHR22Q12	74	0,000	TBC1D10A RAC2 APOL2 SMTN HMOX1 PES1 SUN2 MAFF SELM EMID1 PIK3IP1 TOB2 CSNK1E GATSL3 CBX6 PISD SEC14L2 MYH9 SLC16A8 GAS2L1 GALR3 PDXP APOL5 CACNA1I TOMM22 RBFOX2 PVALB ASCC2 CYTH4 MTMR3 SYN3 HORMAD2 SLC5A4 PDGFB CHEK2 OSBP2 DMC1 APOL6 KREMEN1
CYTOKINE_ACTIVITY	94	0,000	CXCL5 CXCL1 CSF3 CXCL3 CXCL6 IL8 CCL7 CSF2 CCL20 CCL11 CXCL2 CCL5 CCL8 GDF15 NAMPT IL19 TRIP6 CCL24 CDK5 TGFB2 CSF1 VEGFA MIF CCL23 CCL2

Interestingly, HSCs exhibited significantly higher levels of neuronal markers such as NGF, neurotrophin 3, NDNF, and NEDD 2 & 9 than ADHLSCs (Figure 2C).

Such significant differences were also confirmed using immuno-cytochemistry. Indeed, we observed that NCAM is expressed in HSC but not in ADHLSC; a difference stably maintained over the passages

(Figure 3A). Nestin and Desmin expression was predominant in HSCs as compared to ADHLSC (Figure 3A).

HSC but not ADHLSC expressed hepatic cytokeratins like cytokeratin 18 and cytokeratin 19 at the protein level, as demonstrated using western blotting (Figure 3B).

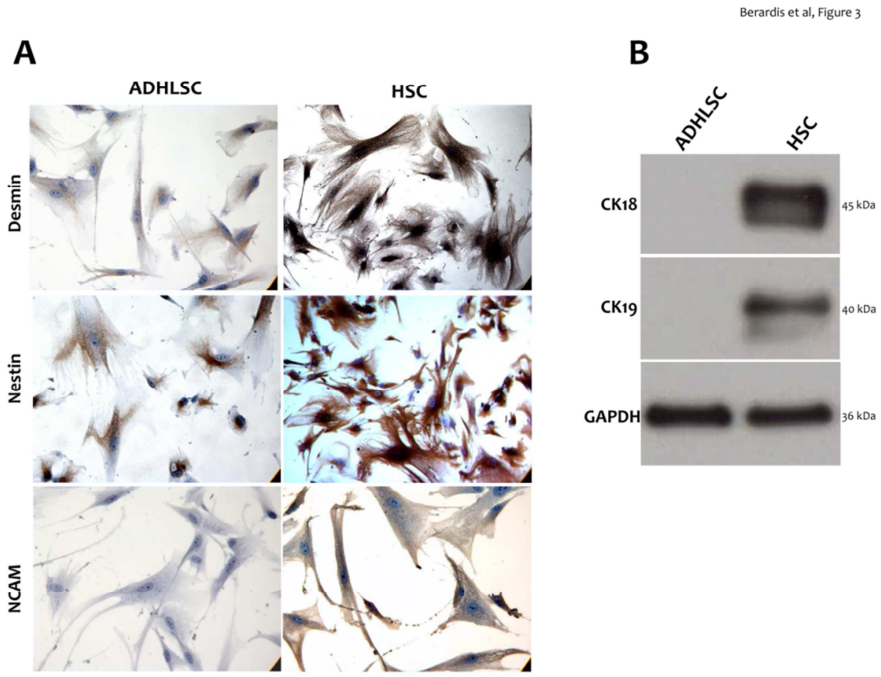


Figure 3. **Immunodetection comparative study.** A, HSCs show a positive immunostaining for neural markers like NCAM and nestin and for desmin. The expression level of nestin and desmin is higher in HSC in comparison with ADHLSC. ADHLSC do not express NCAM at the protein level. Images are representative of several fields examined from four different donors. Magnification 400x. B, Total

proteins were extracted from ADHLSC and HSC and CK-18 and CK-19 immuno-detection was realized using western blotting. Data shown are representative of 4 different cell samples isolated from 4 different donors.

These observations are in accordance with the microarray analyses that revealed a higher expression level of keratins 7, 14, 18, 19, & 34 by HSC when compared to ADHLSCs (Figure 2C).

All together, these findings clearly show that cultured HSCs and ADHLSCs are two different cell populations with distinct gene expression profiles.

Functional characterization of ADHLSCs and HSCs – Cytokines and growth factors secretion

After phenotypic and genotypic characterization, we carried out a functional analysis of both cell populations by assessing their secretome profile in the corresponding conditioned culture medium of cells originated from 3 different donors at passages 5 &7. This was performed on supernatants collected 24 hours after incubation with serum free medium. As collagen is known to be secreted by activated HSCs, we evaluated its secretion by measuring the pro-collagen type-I

C-Peptide in the culture supernatants. No significant difference was observed between HSCs and ADHLSCs (Figure 4A). We thereafter looked at some markers known to play important functional roles in terms of immuno-modulation and liver regeneration. Secretion of Transforming growth factor-beta 1 (TGF-β1), one of the most powerful pro-fibrotic cytokines and involved in inflammatory and immune responses, was confirmed in our activated human hepatic stellate cell cultures. We demonstrated that ADHLSCs secrete equivalent amounts of TGFβ1 in the culture supernatant (Figure 4B).

HGF is a hepatocyte mitogen, which has several crucial physiological functions including organ protection and regeneration. Following liver injury, HGF is known to be secreted by distant organs such as spleen, lungs and kidneys as well as by sinusoidal cells such as Kupffer cells and HSCs. Moreover, HGF has anti-inflammatory properties. We observed a significant difference in HGF production between HSCs and ADHLSCs. The liver stem/progenitor cells seemed to secrete HGF about three times more than HSCs (Figure 4C).

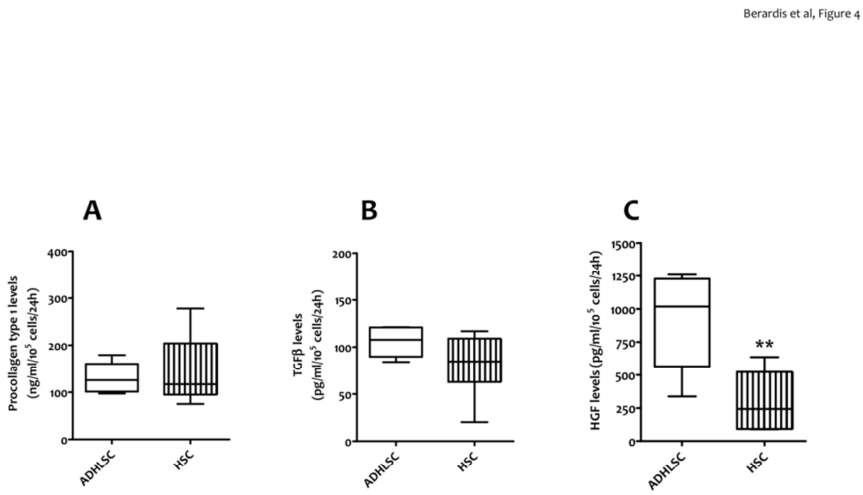


Figure 4. Secretome profile in the conditioned culture medium of ADHLSC and HSC. The Elisa analyses were performed on supernatants (n=3 including the diseased donor, analyzed at passage 5 and passage 7) collected 24 hours after incubation with serum free medium. *A*, the collagen secretion was evaluated by measuring the procollagen type-I C-Peptide, precursor of the collagen type I, by Elisa. No significant difference was observed between HSC and ADHLSC. *B*, TGFβ1 secretion; ADHLSC and HSC secrete an equivalent amount of TGFβ1 in the culture supernatant. *C*, HGF secretion; we observed a significant difference in HGF secretion between HSC and ADHLSC. The liver stem/progenitor cells seem to secrete HGF about three times more than HSC.

In the second part of this functional analysis, we focused on cytokines implicated in the inflammatory response. Indeed, HSC are known to secrete several pro-inflammatory cytokines while ADHLSC seem to

exhibit immuno-modulatory properties. Twenty-seven cytokines were analyzed, using a multiplex technology. The cytokines were classified as growth factors, chemokines, pro-inflammatory cytokines, anti-inflammatory cytokines and those with dual roles. Among the growth factors analyzed, the main differences were noticed for VEGF and PDGFbb for which significant higher levels were detected in ADHLSCs (Figure 5A). The increase in VEGF and PDGFbb levels was respectively 17 and 1.5 times as compared to HSCs from the same donor. Regarding the chemokines analyzed, Eotaxin (CCL11) was 14 times more secreted by ADHLSC than HSC (Figure 5A). With respect to pro-inflammatory cytokines, the major differences between the two cell populations were IP-10 (3.6 times), IL-5 (2 times), IL-7 (2 times), IL-8 (30 times), and IL-17 (4.5 times), which are highly secreted by ADHLSC. To a lesser extent, the same trend was observed for IL-9 (1.6 times), IL-12 (1.7 times), interferonγ (1.5 times) and TNFα (1.6 times) (Figures 5A & 5B). Concerning the anti-inflammatory cytokines, a significant higher level of IL-13 (2.1 times) and IL-10 (1.8 times), was secreted by ADHLSCs as compared to HSCs. IL-1ra, a natural inhibitor of the pro-inflammatory effect of IL1β, and IL-4 (presenting anti- and pro-inflammatory properties) were also produced in higher concentrations by ADHLSCs,

even if the statistical difference was less important (Figure 5B). IL-2 is considered to play a dual role as it has pro- and anti-inflammatory activities. The concentration of this cytokine was found to be higher in the culture supernatant of ADHLSCs (1.5 times) in comparison with the one of HSCs (Figure 5B).

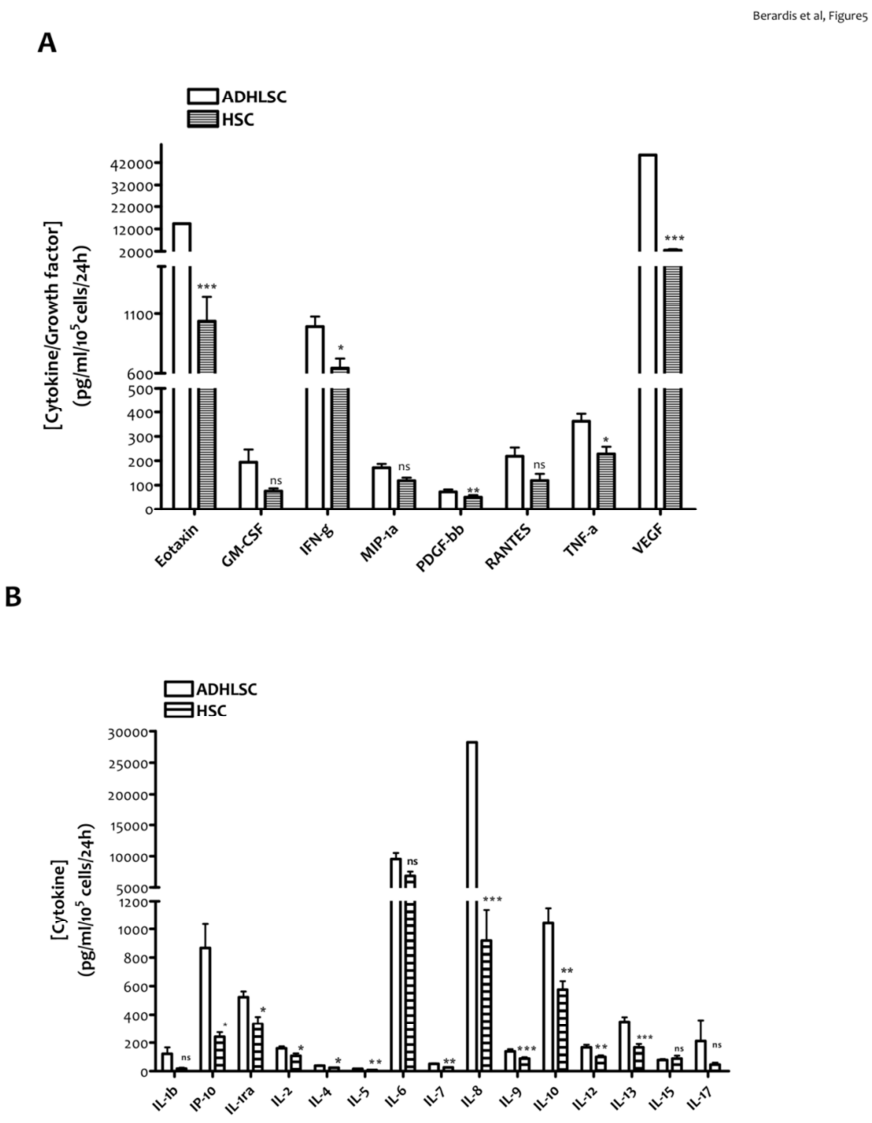


Figure 5. Secretome analysis of ADHLSC and HSC using a multiplex technology (Luminex). The analyses were performed on culture supernatants collected 24 hours after incubation with serum free medium (n=3, from 2 healthy and 1 diseased donors; analyzed at passage 5 and passage 7). The growth factors and cytokines concentrations were calculated for 100 000 cells. *A*, ADHLSC secrete high levels of VEGF, PDGFbb and Eotaxin as compared to HSC. *** denotes a p value <0,001; **

denotes a p value < 0,01; * denotes a p value < 0,05. NS: Not significant. *B*, concerning pro-inflammatory cytokines, ADHLSC highly secreted IP-10 (3.6 times), IL-5 (2 times), IL-7 (2 times), IL-8 (30 times), and IL-17 (4.5 times) as compared to HSC. With respect to anti-inflammatory cytokines, ADHLSC significantly secreted higher level of IL-13 (2.1 times) and IL-10 (1.8 times). *** denotes a p value <0,001; ** denotes a p value < 0,01; * denotes a p value < 0,05. NS: Not significant.

These findings confirm the singularity of both cell populations and support the expression profile differences demonstrated using gene expression analyses.

Discussion

The aim of the current study was to establish an in-depth comparison of ADHLSCs and HSCs, cells of human liver origin exhibiting similar fibroblastic morphology and both reported as stem/progenitor cells. By comparing these cell populations successfully isolated from the same donors, we i) demonstrate that ADHLSC and HSC are two distinct liver cell types displaying significant differences in gene expression profiles as well as functional features, and ii) confirm the stem/progenitor properties of ADHLSC.

Using flow cytometry and immuno-cytochemistry, we confirmed the mesenchymal phenotype of both ADHLSCs and HSCs. Both cell

populations were also negative for hematopoietic and epithelial protein markers rendering the demonstration of their singularity difficult.

To assess a thorough analysis of both cell types, we used microarray analysis and screened more than 10.000 genes on the 4 different samples of ADHLSC and HSC. Top 100 differentially expressed genes were identified. The main differences between ADHLSCs and HSCs are seen at the level of the chemokines expressed. Such chemotactic molecules which regulate the infiltration of immune cells to sites of inflammatory injuries, are initially all expressed in the liver. With respect to the first family, CCL7 seems to be the highly expressed one in ADHLSC in accordance with its documented role in development as demonstrated in neuronal differentiation [17]. CCL7 was also involved in the pro-inflammatory responses that may stimulate liver regeneration at the cellular level [18].

The second CXC chemokine family displays well-documented neutrophil chemotactic, angiogenic, and mitogenic properties [19] [20]. The increased expression levels of CXCL1 and CXCL6 detected in ADHLSC are in accordance with the parallel up-regulated level of IL-8 and LIF. All these cytokines are directly involved in chemotaxis, cell movement and migration of mesenchymal stem cells [21].

HGF, a pleitropic cytokine of mesenchymal origin, participates in the regulation of proliferation, differentiation and chemotactic migration of mesenchymal stem cells [22] [23]. Its high expression level in ADHLSCs is in accordance with their stem/progenitor features as documented in other MSCs. Conversely, HGF levels are abnormally decreased in fibrosis settings which correlate with induced proliferation of activated stellate cells [24]. In the same context, HGF over-expression induces a remarkable anti-fibrotic effect [25] [26]. The anti-fibrogenic effects of HGF have been attributed to the inhibition of the TGFβ pathway, the inhibition of other fibrogenic cytokines and to the induction of matrix metalloproteinases [27] as well as up-regulation of miR-29 thereby regulating collagen expression [28]. This result may suggest potential anti-fibrotic properties of ADHLSC as demonstrated for other mesenchymal stem cells [29] [30].

In our study, we did detect protein expression of cytokeratins 18 & 19 in only HSC as demonstrated using western blotting which is in accordance with gene expression profiling data. The data are supported by the increased expression of desmoplakin, which anchors these intermediate filaments to desmosomes [31]. However, no data is

currently available regarding desmoplakin expression and its role in activated HSCs.

After its interaction with dystroglycan, dystrophin forms a glycoprotein transmembrane complex required for spatial organization of laminin on the cell surface and for basement membrane assembly. Such complex is up regulated during spontaneous activation of HSC in culture [32]. This is also the case for elastin, a marker of maturity of liver fibrosis that was logically up regulated in HSC as compared to ADHLSC [33].

MMP3 (stremolysin), which degrades collagen types II, III, IV, IX, and X, proteoglycans, fibronectin, laminin and elastin, was the highly up-regulated MMP in ADHLSC compared to others MMPs like MMP2 and MMP14. In contrast, MMP3 is down-regulated in other MSCs like adipose-tissue MSC, as compared to fibroblasts [34]. HSCs are known to express both mesenchymal and neural cell lineage markers [35]. Indeed, an increasing number of neural/neuroectodermal markers have been documented in HSCs like NCAM, nestin and NGF [36] [37] [38]. In our study, we confirmed this specificity of HSCs using both microarray and immunocytochemistry. At the protein level, we demonstrated that NCAM is highly expressed in HSCs as compared to ADHLSC (although no gene expression level difference was seen between both cell types).

Nestin expression presents the same profile as NCAM except that its protein expression is detectable in ADHLSC. Indeed, this intermediate filament has been described in mesenchymal stem/progenitor cells [39]. These data are in accordance with the described genetic profile of other MSCs, for which neural markers are down-regulated in comparison with fibroblasts [34]. Together, these phenotypic and genotypic characterization analyses confirmed the singularity of ADHLSCs and HSCs with respect to chemokines, intermediate filaments and neural markers expression levels.

The functional analysis of ADHLSCs and HSCs allowed us to support the hypothesis that both cell populations also behave differentially in basal conditions. Obviously, the secretion profile of each cell type is different and supported the microarray data, emphasizing that ADHLSCs secrete cytokines of therapeutic and immuno-modulatory importance.

In the liver, TGF-β is initially produced in non-parenchymal liver cells, whereas absent in fully differentiated epithelial cells [40]. TGF-β is also a potent activator of myofibroblast differentiation, ECM synthesis, migration, and oxidant production in mesenchymal cells [41]. TGF-β activates HSC, stimulates their ECM secretion and inhibits their apoptosis, which is in accordance with its pro-inflammatory effects

[37]. We recently demonstrated the immuno-modulatory effects of ADHLSC and TGF-β may participate to these effects by inhibiting lymphocytes proliferation, macrophages activation and immunoglobulins secretion [42] [43].

Collagen is one of the predominant structural proteins of the liver extracellular matrix and is involved in stem cell proliferation and differentiation [44] [45] [46]. Accordingly, a comparable secretion level was measured in both analyzed cell types. We also focused on cytokines implicated in the immune response and demonstrated that ADHLSC secreted greater amounts of pro-inflammatory cytokines like IL-7, IL-8, IL-9, IL-12, interferonɣ and TNFα and of some anti-inflammatory cytokines like IL-1ra, IL-4, IL-10 and IL-13. All these cytokines are secreted by adipose-derived MSC, stem cells possessing interesting immuno-modulatory properties [47], a feature recently also demonstrated in ADHLSC [42]. In addition, interferon-ɣ, even with pro-inflammatory properties, has been described as an inhibitor of collagen deposition and HSC activation in vitro and in vivo [48].

These secretion profile analyses also suggest that ADHLSC secreted cytokines and growth factors may be of therapeutic and immuno-

modulatory importance in the context of their transplantation development.

VEGF has been shown to promote liver fibrosis by stimulating the activation, proliferation, and chemoattraction of HSCs [49] [50] [51]. In our study, we demonstrated that VEGF is highly secreted in ADHLSCs as compared to HSCs, which is in accordance with its involvement in wound healing [52] and regeneration process including for the liver [53] [54] [55]. VEGF has also been documented to be involved in supporting stem cell proliferation and survival [56].

In conclusion, our study demonstrates that ADHLSCs and HSCs represent two distinct hepatic cell populations. Even if they share numerous similar characteristics, we highlighted significant differences supporting their singularity mainly at the gene expression and secretion profile levels. This study also provides additional features supporting the potential therapeutic development of ADHLSC for liver cell-based therapy.

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Paper 2

Human liver mesenchymal cells inhibit human hepatic stellate cell activation and induce their secretion of anti-fibrotic molecules via HGF mediated paracrine effect.

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Submitted.

HSC are considered to be the main cell type implicated in the extracellular matrix deposition by undergoing an activation process after liver injury. This activation process, characterized by an increased proliferation rate and an increased collagen secretion level, plays a key role in the development of liver fibrosis. Isolated HSC are able to activate in vitro after only being in contact with the plastic substrate of the culture dishes. Characteristics exhibited by HSC post-plating are similar to those described in vivo.

In the second part of this thesis work, we investigated whether ADHLSC were able to inhibit the in vitro activation process of human stellate cells. The effects of ADHLSC on HSC were evaluated in indirect co-

culture systems and concerned proliferation rate, produced collagen level and anti-fibrotic molecules secretion.

Our data revealed that ADHLSCs were able to significantly inhibit HSC proliferation and their collagen production/secretion. This study shows the potential of ADHLSC to modulate the HSC secretome quality, in particular via the secretion of HGF.

Abstract

OBJECTIVE: Progressive liver fibrosis leads to cirrhosis and end stage liver disease. Controlling stellate cell activation should prevent such evolution. In vivo studies have reported benefit of mesenchymal stem cell therapy to control fibrosis in rodent models, but not yet in human, and in vitro proof of concept using human cells remains scarce. Our aim was to evaluate the in vitro effect of a specific sub population of mesenchymal stem cells harvested from the human liver, the Adult Derived Human Liver Mesenchymal stem/progenitor cells (ADHLSCs) on the activation and secretome profile of autologous and allogeneic human Hepatic Stellate Cells (HSCs).

DESIGN: Indirect co-culture systems used different concentration ratios of ADHLSCs and HSCs, or the conditioned ADHLSC culture media. . HSC number and viability were evaluated by microscopy and biochemical assays whereas proliferation was analyzed using flow cytometry and immunocytochemistry. The HSC secretion profile was evaluated by Elisa and Luminex.

RESULTS: HSC proliferation was inhibited from 24 hours to 7 days when co-cultured with ADHLSCs or their conditioned medium. Plating

was delayed, and HSCs were blocked in G0/G1 phase, with decreased Ki-67 positivity. Pro-collagen I production was reduced and an increased secretion of HGF, IL-6, MMP1 & MMP2 by HSCs was measured. Neutralization of HGF partially blocked the inhibitory effect of ADHLSCs on HSC proliferation and secretion profile.

CONCLUSION: These data provide mechanisms for the anti-fibrotic potential of ADHLSCs, opening the prospect of clinical trials in liver fibrosis.

Introduction

Liver fibrosis control remains an unmet medical need. It occurs in response to chronic liver injury and leads to cirrhosis and end stage liver disease (1;2). Patients require liver transplantation and face long waiting time with progressive disabilities (3). Progressive fibrosis of the graft may also occur after successful transplant (4). Despite the improved understanding of mechanisms underlying liver fibrosis development, the efficacy of most drugs has not been proven in humans (1;5).

Hepatic stellate cells (HSCs) are the main extracellular matrix-producing cells. Following liver injury, HSCs undergo an activation process characterized by the adoption of proliferative, contractile and fibrogenic myofibroblast features (6), a process that can be reproduced in culture plastic dishes (7). Ideal anti-fibrotic therapy should modulate HSC activation, inhibit collagen synthesis and enhance matrix degradation (8).

Mesenchymal stem cells (MSCs) are proposed to treat fibrosis, on the basis of their hepatocyte differentiation and regeneration potential as well as their immunomodulatory properties (9-11). The Adult Derived

Human Liver Stem Cell is a subtype of MSC obtained following collagenase digestion of the liver, with a preferential hepatocyte differentiation pattern (12) (13). MSCs, including ADHLSCs, can produce growth factors and cytokines able to suppress the inflammatory responses (14;15). Moreover, MSCs have the potential to exert a suppressive effect on immune cells (16).

Extra-hepatic MSCs were reported to reverse fibrosis in animal models of induced liver fibrosis (17;18), with a frequent improvement of the hepatic function. The underlying mechanisms are not yet understood. In vitro models are therefore required to better understand how HSC activation can be modulated by MSCs, and their paracrine properties (19-21). Ideally, these systems should use human derived cells as rodent cells may behave and react differently.

ADHLSCs engraft and differentiate in hepatocyte like cells in rodents, and have been infused safely and successfully in children with inborn errors of liver metabolism (10) (11) (22) (23).

In the current study, we investigated the ability of ADHLSCs to modulate the activation of autologous and allogeneic human stellate cells (HSCs) in vitro in an indirect co-culture system.

Materials and methods

ADHLSC and HSC isolation and culture

The protocol and experiments were approved by the ethical committees of St-Luc Hospital and Medicine faculty of Université Catholique de Louvain. An agreement from the Belgian Ministry of Health was obtained for Hepatocytes and Hepatic Stem Cells isolation and banking. A written and signed informed consent has been obtained for each human liver used in the current study (n=4, Table 1).

Table 1: Characteristics of the four liver donors used for the isolation of both HSC and ADHLSC studied in the current work.

Donor number	Age	Gender	Reason of death or liver transplantation	Blood group	Ischemia time
89	3 days	M	Respiratory	A+	4 hours
93	2 years	F	Metabolic disease (Liver transplanted)	O+	1 hour 43
98	7 days	M	Cardio-respiratory arrest	O-	4 hours 20
105	46 years	F	Trauma	B+	9 hours 43

Each liver was isolated using a two-step collagenase perfusion. The obtained crude liver cell suspension was submitted to filtration and low speed centrifugation. The recovered pellet containing liver cells of the parenchymal fraction (mainly hepatocytes) was submitted to primary culture. ADHLSCs were subsequently obtained as previously described (12;15). The supernatant containing the corresponding non-parenchymal fraction was submitted to a Nycodenz gradient (Myegaard, Oslo, Norway) centrifugation step in order to isolate HSCs (24).

Both liver cell types were cultured using DMEM containing 4.5g/L glucose (Life Technologies) supplemented with 10% Fetal Calf Serum (FCS) (Life Technologies) and 1% Penicillin/Streptomycin (Life Technologies), at 37°C in a fully humidified atmosphere (5% CO2) as previously described (15). When reaching 80% confluence, cells were lifted with 0.05% Trypsin-EDTA (Life Technologies) and replated at a density of 5000 cells/cm^2. The viability of recovered cells was evaluated using trypan blue exclusion assay at each passage.

Co-culture systems

In order to evaluate the interactions between ADHLSCs and activated HSCs, indirect co-culture Transwells were used. HSCs were seeded in the lower chamber of a 6-well plate at a density of 10.000 cells/cm^2 while ADHLSCs were placed on collagen-coated membrane inserts (24 mm diameter, 0.4 µm pore size; Corning), with ADHLSC/HSC ratios of 1/100, 1/10, 1/1 and 0/1. The impact of ADHLSCs on HSCs was evaluated by collecting HSCs at 1, 4 and 7 days for analysis. Cell number and viability were evaluated using microscopy and Trypan blue exclusion assays.

Biochemical evaluation of HSC number

Sensitive colorimetric assay Cell Counting Kit-8 (CCK-8) (Sigma) allows the determination of the number of viable cells, following the reduction of the tetrazolium salt WST-8 by the cell dehydrogenases, giving rise to a yellow colored product (Formazan). The amount of Formazan produced is directly proportional to the number of viable cells.

After co-culture, HSC were lifted with 0.05% Trypsin-EDTA (Life Technologies). For each condition, 100 µl of the cell suspension were added into each well of 96-well plates and incubated for 15 minutes at

37°C in a fully humidified atmosphere (5% CO2). A calibration curve using determined viable cells amounts was performed for each experiment. Ten µl of the CCK-8 solution were added per well and the plates were incubated for 2 hours at 37°C in a fully humidified atmosphere. Absorbance was thereafter measured at 450 nm using a Victor X4 plate Reader (Perkin Elmer).

Flow cytometry

For cell cycle analysis, HSCs were lifted with 0.05% Trypsin-EDTA (Life Technologies) after co-culture. Two hundred thousand cells were used for each condition. Cell suspensions were centrifuged, washed twice with PBS, fixed with 700µl of cold ethanol and incubated for 30 minutes on ice. HSC were then washed again with PBS before being incubated with 100 µg/ml Propidium Iodide (Life Technologies), 0.1 mg/ml RNase (Sigma) and 0.1% Triton X-100 (Sigma) for 30 minutes at 37°C and then for 15 minutes on ice, before reading with a CANTO II flow cytometer. The analyses were performed using the BD FACSDiva Software. The different phases of the cell cycle were determined by measuring the area under the curve using the FlowJo Software.

Immunocytochemistry

After 24 hours incubation with ADHLSC or HSC conditioned medium in 24 well plates, cells to analyze were fixed using paraformaldehyde 3.5%, for 15 min at room temperature. Endogenous peroxidase was eliminated using hydrogen peroxide 3.3% for 3 minutes. All steps were performed at room temperature. Cells were permeabilized using PBS containing 1% Triton X-100 (Sigma) for 10 minutes. Non-specific immuno-staining was prevented by 1h incubation in PBS containing 1% Bovine Serum Albumin (Sigma). Thereafter, cells were incubated with Ki-67 antibody (Dako) at a concentration of 1/150 for 1h. After washing, cells were incubated with secondary antibody (EnVision – Dako) during 30 minutes. Revelation was performed after 5 minutes incubation with liquid DAB and chromogen substrate (Dako). Counterstaining was performed using Mayer's hematoxylin for 10 minutes. Preparations were then mounted for microscopic analysis (DMIL, Leica, Belgium). For each experimental condition, four different fields were analyzed and the stained / unstained nuclei were counted using the ImageJ Software for a total of 2500 nuclei.

Apoptosis assay

Apoptosis of HSC was determined using FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen). The experiments were performed according to the manufacturer's instructions. Briefly, HSCs were harvested, washed with PBS and resuspended in 100µl of Annexin V binding buffer before adding 5µl of Annexin V and 5µl of Propidium iodide. After an incubation of 15 minutes in the dark and the addition of 400µl of Annexin V binding buffer, cells were analyzed by flow cytometer (Canto II, BD). Cells treated with 1mM Hydrogen Peroxide were used as positive control for apoptosis.

ELISA

After removal of the inserts containing ADHLSC and medium aspiration, recovered HSC were washed with sterile PBS and a serum-free medium (DMEM containing 4.5g/L glucose (Life Technologies)) was added for 24 hours. Thereafter, supernatants were collected and cells were lifted for counting and evaluation of viability. Collagen secretion was evaluated using an Elisa kit for the procollagen type I C-Peptide (Takara Bio Inc, Japan). The experiments were performed according to the manufacturer's instructions. The measurement of the absorbance

at 450 nm was done with a Victor X4 plate Reader (PerkinElmer). The results were randomized according to the number of cells collected.

Luminex analysis

The secretome of HSCs after 24 hours of co-culture was evaluated using a 9-plex kit (TNFα, MMP-9, MMP-2, Interferon ɤ, HGF, IL-6, MMP-1, IL-10 and MMP-13) (R&D Systems) and the Luminex technology (Bio-Plex 200, Biorad). The supernatants were prepared as previously described for the pro-collagen type I C-Peptide Elisa. The principle of the technique is based on color-coded beads, which enable the detection of up to 100 cytokines simultaneously. The primary antibody directed against the target protein is conjugated with the dyed beads. After several washes to remove unbounded proteins, a secondary biotinylated antibody is added to the reaction. Streptavidin-phycoerythrin (Streptavidin-PE) is then added to bind the biotinylated antibody. By measuring the relative fluorescence intensity, the antigen-antibody reaction can be evaluated. The assays were performed following the manufacturer's instructions.

Briefly, after the pre-wetting of the plate, 50µl of the beads were added into each well. Fifty µl of the samples were then added into each well

and the plate was incubated for 2 hours at room temperature on a plate shaker at 200 rpm. The plate was then washed three times with the Wash Buffer and 50 µl of the diluted Biotin antibody was added to each well. After an incubation of 1 hour at room temperature with shaking, the plate was washed three times before adding 50 µl of the Streptavidin-PE solution into each well. Finally, after an incubation of 30 minutes on a plate shaker at 200 rpm and three washes, the beads were resuspended with 100µl of Wash Buffer into each well. After an incubation of 2 minutes at room temperature on a plate shaker at 200 rpm, the plate was read by the Luminex machine, and the data were analyzed using Bio-Plex Manager 6.0.

HGF neutralization

Neutralization of HGF was performed using 20µg/ml anti-HGF antibody (R&D Systems), diluted in the conditioned medium. The conditioned medium without anti-HGF served as control. The antibody was incubated in the ADHLSC/HSC corresponding conditioned medium for 1 hour at 37°C before being in contact with HSCs. After a 24 hour incubation, HSCs were lifted for counting, viability assay and secretome analysis.

Statistics

Results are expressed as mean ± standard error of the mean (SEM). Statistical differences were determined by paired Student's t-test for two samples analysis and One way ANOVA followed by the Newman-Keuls post-hoc test. Differences were considered significant when p values $*p<0.05$, $**p<0.01$, $***p<0.001$.

Results

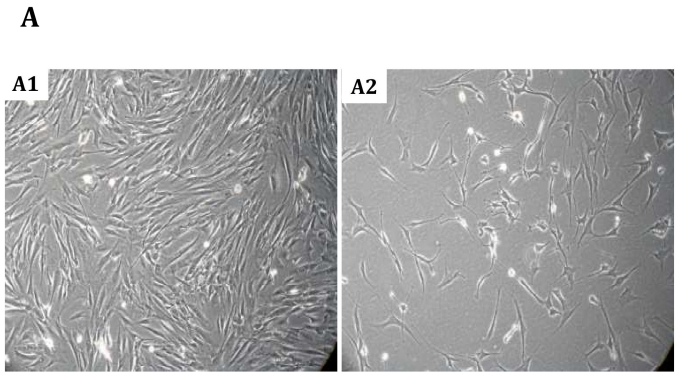
The decrease of HSC recovery induced by ADHLSCs is associated to an inhibition of both plating and proliferation

We investigated the influence of ADHLSCs on the modulation of the activation process of HSCs. We mainly studied the indirect interactions between both cell types, by using a Transwell co-culture system. For each experiment, both ADHLSCs and HSCs used were obtained from the same human donor liver.

As activated HSCs are characterized by an increased proliferation rate, we first analyzed the influence of ADHLSCs on their recovered number. After plating an initial HSC cell number in the lower chamber, ADHLSCs were placed on the collagen-coated membrane insert with ADHLSC/HSC ratios of 0/1, 1/100, 1/10 and 1/1. Activated HSCs from 4 different donors were collected after 24 hours, 4 days and 7 days of co-culture with ADHLSCs. A significant decrease in the number of recovered HSCs was noted after 24 hours of co-culture. To avoid operator bias, recovered HSC number was independently evaluated and the diminution was confirmed by three different investigators. In

addition, the decrease in the number of recovered HSCs was optimal when the lowest ADHLSC density (1/100 ratio) was used in the co-culture (42 ± 5 % vs 36 ± 6 % vs 81 ± 7 % for 1/100, 1/10 and 1/1 culture conditions respectively) (Figure 1A & 1B). Our data also revealed that the inhibitory effect initiated in the first 24 hours, was maintained after 4 and 7 days of co-culture for all culture conditions (Figure 1 B).

Figure 1



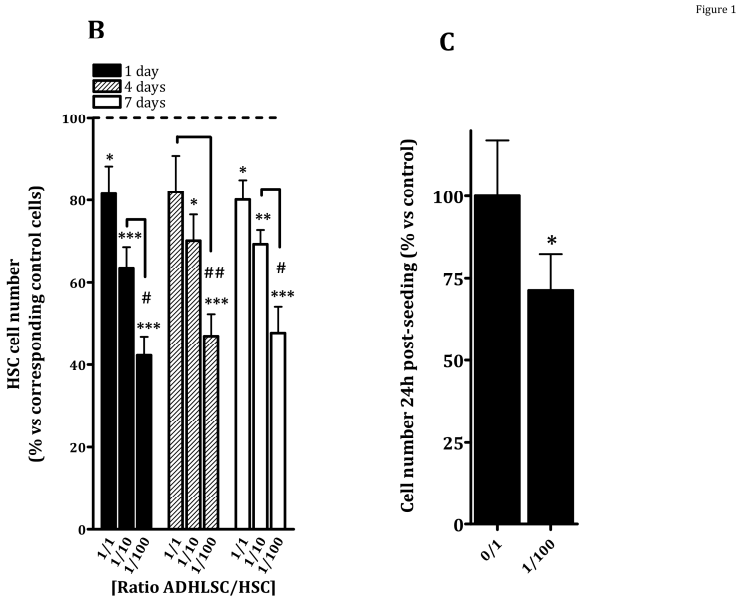


Figure 1: Effect of ADHLSC on HSC culture. A, Morphology of HSC recovered after 24 hours of co-culture with no ADHLSC (A1) or with a ADHLSC/HSC ratio of 1/100 (A2). B, The analysis of the number of adhering HSC collected after co-culture with ADHLSC revealed a significant decrease in HSC number after 24 hours at an ADHLSC/HSC ratio of 1/100. This effect was maintained up to 7 days (n=6 for 24 hours; n=4 for 4 days and 7 days). C, The analysis of HSC number after 24hours of co-culture with ADHLSC using CCK8 biochemical assay revealed a significant decrease in HSC number at an ADHLSC/HSC ratio of 1/100 (n=4). Results are expressed as mean ± standard error of the mean (SEM). *** denotes a *p* value <0,001; ** *p*<0,01; * *p*<0,05.

Several quality control experiments were performed in order to validate the model. Firstly, ADHLSCs were seeded alone in an insert for 24 hours. Thereafter, the ADHLSC containing insert was added to HSCs that were seeded in a lower chamber. After 24 hours co-culture, we did

observe the same decrease in HSC number as with ADHLSC/HSC ratio of 1/100 (data not shown). Secondly, we investigated if this ADHLSC inhibitory effect on HSCs could be potentiated by replacing the insert with one containing freshly trypsinized cells. After 24 hours of co-culture, the addition of the new insert did not induce additional decrease of HSC number as compared to the conditions mentioned above (data not shown).

To strengthen the result we observed after microscopically counting HSCs post-co-culture with ADHLSCs, we analyzed HSC recovered number using the CCK-8 biochemical assay. After 24h, we demonstrated that HSCs co-cultured with 1/100 ADHLSC density showed a 30% decrease of cell number (100 ± 19.7 % vs 71.3 ± 13.0 % for 0/1 and 1/100 culture conditions respectively) (Figure 1C).

Thereafter, we went into further analysis of the mechanisms behind the diminished number of adhering HSCs obtained after 24h co-culture with ADHLSC. To do so, we investigated whether this could be related to deficient plating, inhibition of proliferation and/or induced cell death. We firstly followed the plating of HSC 24h after the initiation of the co-culture. We counted HSCs in the floating fraction and compared

their number to those obtained after enzymatic detachment. When focusing on the counted adhering fraction, we confirmed the decreased HSC number in the 1/100 co-culture conditions (100 ± 9.3% vs 61 ± 6.5% for 0/1 and 1/100 culture conditions respectively). When analyzing the number of HSCs present in the floating fraction, we revealed that it was significantly 4 times higher in the condition for which HSCs were co-cultured with ADHLSCs at 1/100 ratio (Figure 2A). When considering 100% as the sum of the recovered HSCs in both floating and adhering fractions, we demonstrated that HSC number in the floating fraction is increased up to 13.7 ± 0.8 % in the 1/100 co-culture condition as compared to 2 ± 0.4% in the 0/1 group (Figure 2A). In the adhering fraction, we revealed that the 1/100 co-culture condition logically represents 86.4 ± 0.8 % as compared to 97.8 ± 0.4 % in the 0/1 co-culture group (Figure 2A).

Thereafter, we checked whether the same effect may be obtained by using ADHLSC conditioned culture medium. Both ADHLSCs and HSCs (as controls) were seeded separately at the same initial density than in the co-culture conditions. Then, the culture supernatants were collected after 24 hours and freshly trypsinized HSCs were incubated with these

recovered conditioned media for 24 hours. HSCs were then lifted for counting and viability evaluation.

The same effect was observed when HSCs were only incubated with conditioned culture medium of ADHLSCs. Indeed, we demonstrated a similar decrease in the number of adhering HSCs (100 ± 19.1 % vs 62.1 ± 16.4 % for 0/1 and 1/100 culture conditions respectively) concomitantly to an increase in the number of floating HSCs (100 % vs 280 % for 0/1 and 1/100 culture conditions respectively). When considering 100% as the sum of the recovered HSC in both floating and adhering fractions, we observed that HSC number in the floating fraction is increased up to 12 ± 2.1 % in the 1/100 co-culture condition as compared to 3 ± 1.4% in the 0/1 group (Figure 2B). In the adhering fraction, we revealed that the 1/100 co-culture condition logically represents 88 ± 2.1 % as compared to 97 ± 1.4 % in the 0/1 co-culture group (Figure 2B).

The same described effect was obtained when using allogeneic ADHLSCs (data not shown).

We also evaluated the HSC death using flow cytometry and Annexin V - PI staining. We did not observe any difference in cell death induction between HSCs incubated with ADHLSC conditioned medium and those

cultivated with HSC conditioned medium (Figure 2C). The majority of the cells were viable cells as they were negative for both Annexin V and PI staining. Only a small amount of cells were in early (Annexin V^+ / PI^-) or late (Annexin V^+ / PI^+) apoptosis (Figure 2C).

Figure 2

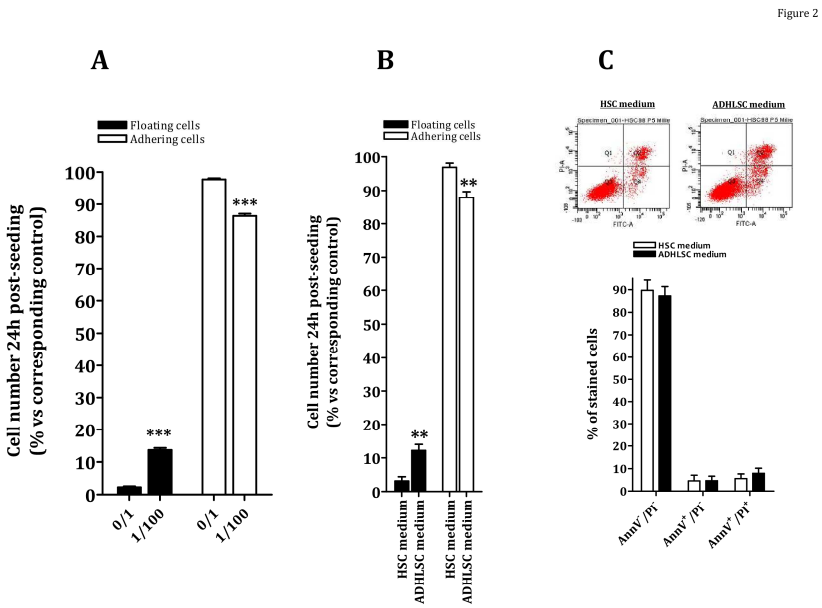


Figure 2: **Effect of ADHLSC on HSC plating and viability.** A, The counting of floating and adhering HSC number after 24 hours of co-culture with ADHLSC showed an increase in the number of floating HSC concomitant with a decrease in the number of adhering HSC at an ADHLSC/HSC ratio of 1/100 (n=4). B, The counting of the number of floating and adhering HSC after 24 hours of incubation with ADHLSC conditioned medium showed similarly an increase in the % of floating HSC and a decrease in the % of adhering HSC, as compared with HSC incubated the same period of time with HSC conditioned medium (n=4). C, Following Annexin V – PI staining, no significant

difference in cell death induction was noticed between HSC cultivated for 24 hours with ADHLSC or with HSC conditioned medium (n=4). Results are expressed as mean ± standard error of the mean (SEM). *** denotes a *p* value <0,001; **p<0,01; * p<0,05.

In order to understand the kinetic of this deficient plating, we analyzed HSC number both in the floating and adhering fractions at different time points post-seeding with either ADHLSC or HSC conditioned medium. Our results demonstrated that the number of HSCs incubated with ADHLSC conditioned medium and then not adhering yet is 2 times higher in the floating fraction as compared to control HSCs, early after seeding (34 ± 5% vs 16 ± 2.9% at 2 hours; 21 ± 5% vs 12 ± 2.9 % at 4 hours) (Figure 3A). This delayed plating remained maintained and optimal after 24h post-seeding (16 ± 2.9% vs 5 ± 1.1 % at 8 hours; 10 ± 2.1% vs 3 ± 1.2% at 24 hours) (Figure 3A). In parallel, the number of adhering cells was also evaluated and confirmed a low HSC number when incubated with ADHLSC conditioned medium as compared to control group (66 ± 4.9% vs 84 ± 2.9% at 2 hours; 79 ± 5 % vs 88 ± 2.9% at 4 hours; 84 ± 2.9% vs 95 ± 1.1% at 8 hours; 70 ± 1.2 % vs 91 ± 2.1 % at 24 hours) (Figure 3B).

Figure 3

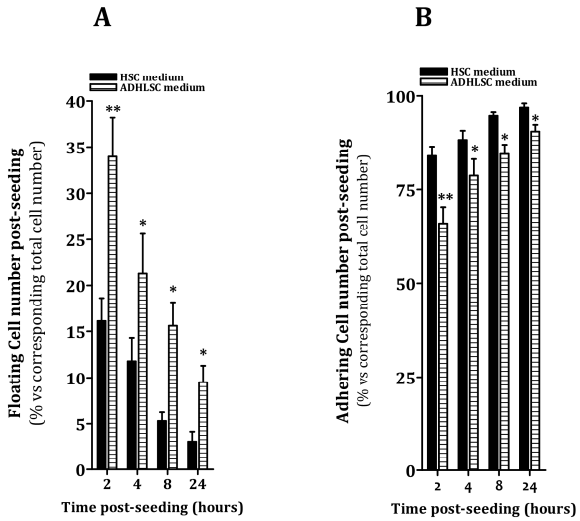


Figure 3: ADHLSC delay HSC post-seeding adhesion. The plating kinetic analysis revealed a higher number of floating HSC (A) and a lower number of adhering HSC (B) at 2, 4, 8 and 24 hours post-seeding in the group of HSC incubated with ADHLSC conditioned medium in comparison with the group incubated with HSC conditioned medium (n=4). Results are expressed as mean ± standard error of the mean (SEM). *** denotes a *p* value <0,001; ***p*<0,01; **p*<0,05.

The evaluation of HSC cycle was carried out with PI staining and flow cytometry whereas the data were analyzed using Flow Jo software. For these results, we demonstrated that 59 ± 4.2% of HSCs co-cultured for 24 hours with ADHLSCs at ratio 1/100 are blocked in G0/G1 phase as

compared to control cells (44 ± 6.5%) (Figure 4A). While no significant difference was seen for S phase, we revealed a significant decrease in the number of HSC in the G2/M phase when co-cultured with ADHLSC (5 ± 2.4%) as compared to control HSC (15 ± 1.7%) (Figure 4A). The same result was obtained when HSCs were incubated with conditioned medium of ADHLSCs both with respect to G0/G1 phase (70 ± 8.9 % for HSC incubated with ADHLSC conditioned medium vs 50 ± 14.5% for HSC incubated with HSC medium) as well as G2/M phase (3 ± 2% when incubated with ADHLSC medium as compared to 11 ± 3.1% when HSCs were incubated with HSC conditioned medium) (Figure 4B). Moreover, we observed the same proliferation inhibition (increase in the number of cells in G0/G1 phase and decrease in the number of cells in G2/M phase) when HSC were seeded during 24 hours before being in contact with ADHLSC conditioned medium (data not shown). In parallel, we used immunocytochemistry for Ki67 protein to confirm flow cytometry data. The number of immunostained nuclei was evaluated on 4 different microscopy fields per donor using the Image J software for a total of 2500 nuclei (Figure 4C). We observed a significant decrease in the number of immunopositive HSC nuclei after an incubation of 24 hours with ADHLSC conditioned medium (23 ± 1.7%) in comparison with

HSCs incubated with HSC medium (46 ± 2.9%) which corroborates with a decrease in the number of proliferating cells.

Figure 4

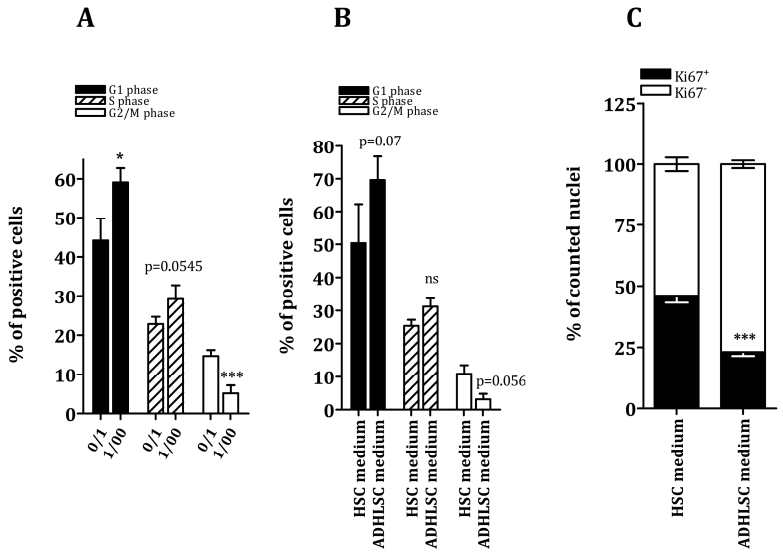


Figure 4: Effect of ADHLSC on HSC proliferation. HSC cycle analysis using PI staining demonstrated an increase in the number of HSC blocked in the G0/G1 phase and a decrease of the number of HSC in the G2/M phase after 24 hours of co-culture with ADHLSC at an ADHLSC/HSC ratio of 1/100 (A) and after 24 hours of incubation with ADHLSC conditioned medium (B) (n=4). C, The Ki67 immunostaining of HSC showed a significant decrease of the number of immunostained nuclei of HSC pre-incubated for 24 hours with ADHLSC conditioned medium, in comparison with the HSC group incubated with HSC conditioned medium (n=4). For each experimental condition, four different fields were analyzed and a total of 2500 nuclei were counted using Image J software. Results are expressed as mean ± standard error of the mean (SEM). *** denotes a *p* value <0,001; **p<0,01; *p<0,05.

ADHLSCs modulate HSC secretion profile by inhibiting pro-collagen type I and stimulating the extracellular release of anti-fibrotic molecules

Upon activation, HSCs are known to increase their secretion of collagen type I, one of the major components of the extracellular matrix. Therefore, we investigated the influence of ADHLSCs on the collagen secretion capacity of HSCs by measuring the pro-collagen I (precursor of the collagen type I) secretion using Elisa assay. HSCs were incubated with ADHLSCs at a ratio of 1/100 for 24 hours. Then, culture medium was discarded and fresh serum free medium was added for an additional 24 hours. The supernatants were collected and adhering HSCs were lifted for counting and viability evaluation. We demonstrated a significant 45% decrease in the amount of pro-collagen I secreted by HSCs when co-cultured with ADHLSCs (210.2 ± 56.8 ng/ml/24h/10^5 cells) in comparison with the control group (384.4 ± 77.2 ng/ml/24h/10^5 cells) (Figure 5A).

Using a multiplex Luminex assay, we also demonstrated that the levels of secreted HGF by HSCs was increased when co-cultured with

ADHLSCs at a ratio of 1/100 (31.6 ± 6.6 pg/ml/24h/10^5 cells) as compared to control HSCs (16.1 ± 4.8 pg/ml/24h/10^5 cells). The same tendency is observed for IL-6 (29.8 ± 15.4 pg/ml/24h/10^5 cells for control vs 66.1 ± 31.7 pg/ml/24h/10^5 cells for HSCs co-cultured with ADHLSCs) (Figure 5B).

Figure 5

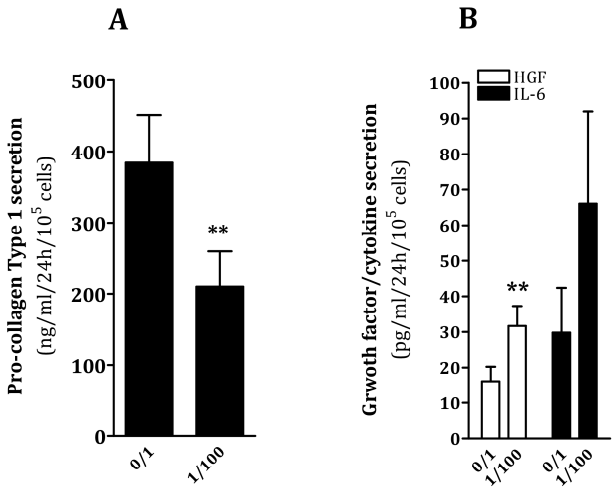


Figure 5: **HSC secretome modulation after 24 hours of co-culture with ADHLSC**. A, ADHLSC inhibit pro-collagen type I secretion by HSC after 24 hours co-culture with ADHLSC at a ADHLSC/ HSC ratio of 1/100, as evaluated by ELISA (n=4). B, ADHLSC increase HGF and IL-6 secretion by HSC after 24 hours co-culture with ADHLSC at a ADHLSC/ HSC ratio of 1/100, as evaluated by multiplex technology (Luminex) (n=4). Results are expressed as mean ± standard error of the mean (SEM). *** denotes a *p* value <0,001; ***p*<0,01 ; **p*<0,05.

The secreted levels of metalloproteinases MMP1 and MMP2, involved in the extracellular matrix degradation, were also increased after 24 hours of co-culture with ADHLSCs (Table 2). This tendency was observed for each donor, even if high inter-donor variability was noticed.

Table 2: HSC MMP secretion level

	MMP1 (pg/ml/24h/100 000 cells)				**MMP2 (pg/ml/24h/100 000 cells)**			
	Donor 1	Donor 2	Donor 3	Donor 4	Donor 1	Donor 2	Donor 3	Donor 4
ADHLSC/HSC ratio								
0/1	474,18	1183,05	1005,03	4550,99	329,02	469,68	1919,75	1959,93
1/100	843,57	1515,79	2248,78	6462,41	466,6	718,12	3288,16	2496,38

Neutralization of HGF reversed the inhibitory effect of ADHLSCs on both HSC number and secretion profile

As previously described, ADHLSCs secrete high levels of HGF, a growth factor known to exhibit anti-fibrotic properties (15). As the inhibitory effect of ADHLSCs on HSC proliferation seems to be mediated by soluble factors, we investigated the potential role of HGF by neutralizing this growth factor. HSCs were incubated with the conditioned medium (from ADHLSC or HSC) supplemented with anti-HGF antibody (20 µg/ml). After 24 hours, we proceeded to HSC counting and viability evaluation. With respect to the floating fraction, we did not see any effect of HGF on the floating HSC population (100 ± 24% for control HSCs; 108 ± 11% for HSCs pre-incubated with anti-HGF antibody; 277 ± 23% for HSC incubated with ADHLSC conditioned medium; 254 ± 22% for HSC incubated with both ADHLSC conditioned medium and anti-HGF) (Figure 6A). Nevertheless, HGF seems more involved in modulating the HSC adhering fraction. Indeed, when anti-HGF was diluted in ADHLSC conditioned medium, it partially inhibited the decrease of HSC number (86 ± 3%) normally detected when HSCs were incubated with ADHLSC conditioned medium alone (64 ± 3%) (Figure

6A). No effect of HGF antibody was noted when incubated with HSC conditioned medium.

We also studied the effect of HGF neutralization on the HSC secretion profile. After a wash-out of 24 hours with serum free medium, we evaluated the HSC pro-collagen I secretion using ELISA. We demonstrated that anti-HGF blocked the inhibitory effect on collagen secretion by HSCs when incubated during the 24 hours co-culture with ADHLSC conditioned medium (Figure 6B). Neutralization of HGF during the ADHLSC/HSC co-culture step also significantly inhibited the HSC secretion of IL-6, HGF, MMP1 and MMP2 (Figure 6C).

Figure 6

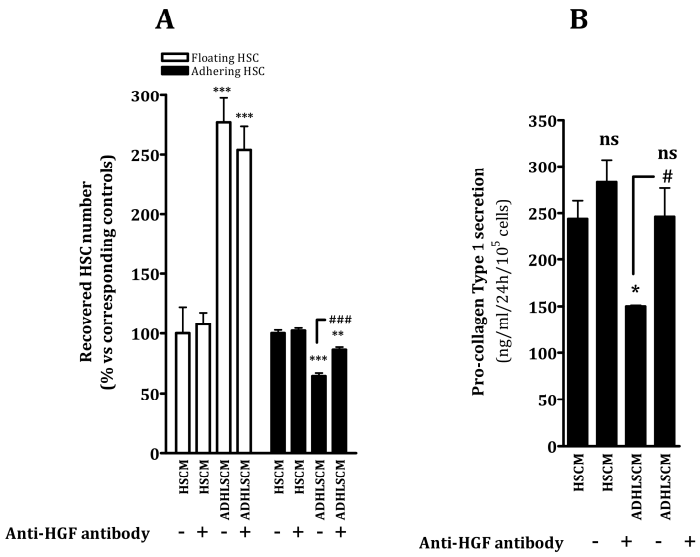

A
Floating HSC
Adhering HSC
Recovered HSC number
(% vs corresponding controls)
300
250
200
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Figure 6

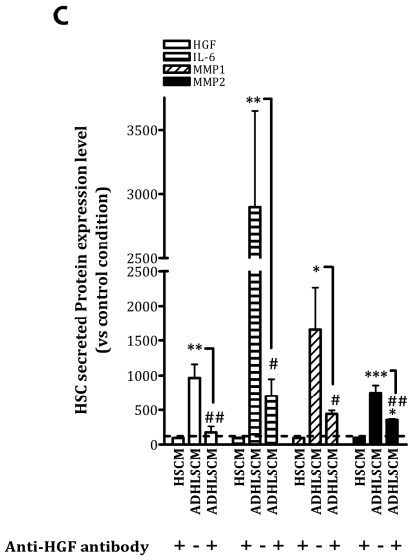

Figure 6: Involvement of HGF in the effect of ADHLSC on HSC proliferation and pro-collagen type I secretion. A, The counting of floating and adhering HSC number after 24 hours of incubation with ADHLSC or HSC conditioned medium was evaluated after pre-incubation with or without anti-HGF antibody. Results showed a significant partial inhibition of the decrease in the number of adhering HSC when incubated with ADHLSC conditioned medium containing anti-HGF ($n \geq 5$). B, Inhibition of the effect of ADHLSC conditioned medium on HSC pro-collagen type I secretion when anti-HGF antibody was added (n=3). C, Anti-HGF antibody significantly inhibited the increase of HGF, IL-6, MMP1 & 2 secretion by HSC when supplemented during their 24 hours co-culture with ADHLSC conditioned medium. The analysis was performed via multiplex technology (Luminex) (n=4). Results are expressed as mean ± standard error of the mean (SEM). *** denotes a *p value* <0,001; ***p*<0,01; **p*<0,05.

Discussion

The novelty of our work is to bring evidence and mechanisms by which human liver derived mesenchymal cells, or their secretome, could be used as anti-fibrotic agents. Existing in vivo studies suggest the potential benefit of mesenchymal stem cells (MSCs) infusion to reverse fibrosis in diseased animal livers (25) (26) (27), and to improve liver function in human liver diseases (28) (29). One study in human has reported histological improvements following intra-arterial infusion of human BM-MSCs in alcoholic patients (30). In vitro, the indirect interaction between human bone marrow derived MSCs (BM-MSCs) and human liver derived stellate cells was investigated; the authors showed that BM-MSCs in transwell co-culture systems inhibit stellate cell proliferation and induce their apoptosis, via nerve growth factor secretion (31). No information was obtained on collagen production by HSCs, nor on MMP and other soluble factor secretion by MSCs or HSCs. In a separate in vitro study using rodent stellate cells, human MSCs reduced HSC proliferation and pro-collagen I production (19).

Our study brings additional clues to the interaction mechanisms involved in man. We selected human liver derived MSCs (ADHLSCs), as these resident cells are more closely related to the liver (12). They display immuno-modulatory properties (32), have a more pronounced differentiation capacity into hepatocyte like cells and participate to liver regeneration (33) (12) (13): these latter characteristics are said to be associated to anti-fibrotic effects (9) (25) (27).

We demonstrated that bioactive factors, constitutively secreted by ADHLSCs can modulate the activated phenotype of autologous and allogeneic human HSCs, inhibit their proliferation and reduce their pro-collagen type I secretion in vitro. Secretion of MMPs were attributed to MSCs in previous in vivo studies (34-36). Our human cell based in vitro model allows to identify HSCs themselves as a source of MMP and other anti-fibrotic molecules. ADHLSCs appear indeed not only to secrete these soluble factors (like HGF) (15), but also to stimulate the secretion of various potential anti-fibrotic molecules by HSCs, i.e. MMP1& 2, HGF and IL6. This suggests the possible co-implication of an autocrine loop of HSCs in the anti-fibrotic process induced by ADHLSCs.

When HSCs are plated in culture plastic dishes, they progressively lose their vitamin A stores, proliferate and increase their extracellular

matrix production, mimicking the in vivo transformation into myofibroblasts.(7).

The inhibitory effect on HSC's proliferation and collagen production was observed when the number of ADHLSCs used was one hundred times lower than the number of plated HSCs. This effect could be reproduced using ADHLSC conditioned medium, both with autologous and allogeneic cells.

The finding that inhibition of HSC proliferation was inversely proportional to the number of ADHLSCs used is a striking difference compared to observations with extra hepatic MSCs (19) (20); this may be explained either by the secretion of an inhibitory molecule by ADHLSCs or HSCs themselves when submitted to higher ADHLSC/HSC ratios, or by a more efficient action of the soluble antifibrotic factor(s) at a determined concentration.

To assess further the mechanism by which HSC proliferation could be inhibited, we blocked the secretion of HGF, one of the factors found to be highly secreted by ADHLSCs (15). HGF neutralization partially reversed the inhibitory effect of ADHLSCs on HSC proliferation. These data are in accordance with published data, describing the ability of

HGF to inhibit the platelet derived growth factor (PDGF) mediated over-proliferation of myofibroblasts (37).

The decrease in the amount of pro-collagen type 1 secreted by HSCs is promising as the collagen type 1 represents the major component of the extracellular matrix produced in excess during the development of liver fibrosis. HGF neutralization also reverted the ADHLSC-induced inhibition of HSC pro-collagen I secretion, in accordance with previous data showing the ability of HGF to inhibit the pro-fibrogenic TGF-β pathway (37). HGF can induce the production of metalloproteinases by lung myofibroblasts (37), and we showed that HSCs also increased their secretion of MMP1 and 2, both involved in extracellular matrix degradation. The increase of the secretion level of IL-6 by HSCs in our model can also be of therapeutic importance as this cytokine is implicated in the regeneration and survival of hepatocytes (38). A recent study demonstrated that IL-6 and murine MSCs synergistically enhanced hepatic repair, improved hepatic function and reduced liver fibrosis in mice (39).

Only a small number of ADHLSCs seem necessary to exert the beneficial inhibitory effect on activated HSCs. The use of allogeneic cells is conceivable as the modulation of HSC activation was efficiently

reproduced with allogeneic ADHLSCs. These findings seem potentially attractive for the development of a cell-based therapy dedicated to liver fibrosis. The maximal effect was observed for a ratio of 1 ADHLSC /1OO HSCs. According to the number of stellate cells in the human liver, the quantity of ADHLSCs required to treat a patient would be 240 million cells, a quantity that can easily be infused in a patient (10) (11;40).

In conclusion, we show the capacity of human liver derived MSCs (ADHLSCs) to modulate the activation of human HSCs in vitro, via a paracrine mechanism. Inhibition of HSC proliferation and pro-collagen secretion as well as up-regulated secretion of anti-fibrotic molecules by HSCs, seem to be the likely involved mechanisms. These effects seem to be mediated at least in part by HGF highly secreted by ADHLSCs. ADHLSCs and/or their secretome are therefore candidates for liver fibrosis treatment.

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Evaluation of the anti-fibrotic properties of ADHLSC in an animal model of induced liver fibrosis: preliminary results

The in vitro results generated in the current thesis allow us to consider the potential use of ADHLSCs for the treatment of liver fibrosis.

In vivo studies are currently ongoing and may provide additional and exploitable information regarding the benefit of ADHLSCs in a fibrotic liver environment.

In order to address this issue, we transplanted undifferentiated ADHLSCs in SCID Beige mice in which liver fibrosis was induced by chronic intraperitoneal injections of CCl4, a widely used toxic. The mentioned animal model is well standardized and characterized according to the literature. A centrolobular necrosis followed by a wound healing response is generated after the transformation of CCl4 into a toxic radical $CCL3^{-}$ by CYP2E1. Significant fibrosis develops 2-4

weeks after CCl4 administration. Severe bridging fibrosis occurs after 5-7 weeks, cirrhosis after 8 to 9 weeks whereas portal hypertension and ascites progressively appear after 10-20 weeks [1] [2].

The immunosuppressed SCID Beige mice were used is the current experiments because they i) lack NK cells, B and T lymphocytes; NK cells displaying anti-fibrotic properties [3], ii) develop an hepatic fibrosis comparable to wild-type mice [4].

Seven-week-old-mice were injected twice a week with CCl4 (0.5 ml/kg) during 3 weeks, in order to induce the development of liver fibrosis. Fourty-eight hours after the sixth injection of CCl4, one million of suspended ADHLSCs was intrasplenically injected (n=12)and CCl4 injections were carried on for two additional weeks. Control sham mice received an intrasplenic injection of the vehicle (PBS/NAC) (n=5). Mice were sacrificed 48 hours after the last injection of CCl4 after which blood and liver samples were recovered. The fibrosis level was evaluated on liver slices using Sirius Red staining whereas the activated murine hepatic stellate cells were identified with ASMA immunostaining. The gene expression of murine fibrosis markers

(ASMA and collagen I) was evaluated using RT-qPCR. Finally, the level of ALT was measured in the serum of the sacrificed mice, in order to evaluate their liver function. The experimental protocol is represented in figure 1.

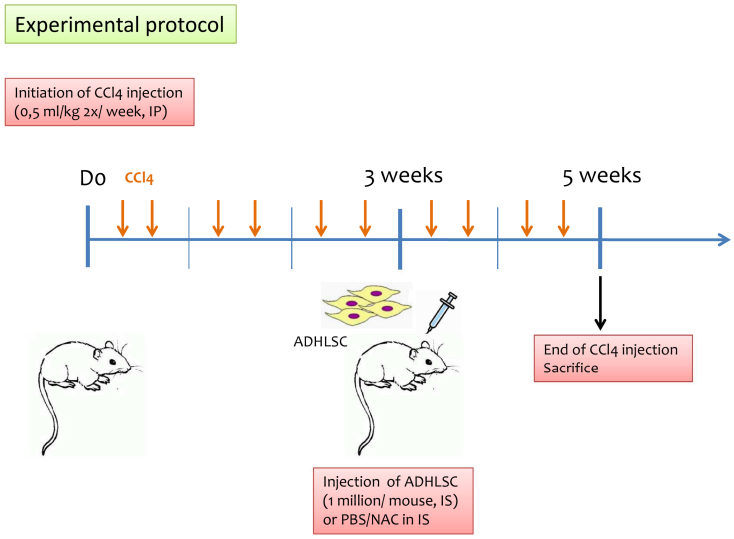

Figure 1: Experimental protocol of liver fibrosis induction and of ADHLSC injection in SCID Beige mice.

Preliminary results showed an improved survival rate in the group of mice treated with CCL4 and transplanted with ADHLSCs, as compared to CCL4 treated and sham operated ones. The mortality in the groups previously treated with CCl4 can be explained by the fact that the dose of Temgesic (136 µg/kg) given during the postoperative care was too high for mice presenting an hepatocellular insufficiency. This hypothesis was confirmed by a second experiment in which a lower dose of Temgesic (22.5 µg/kg) was used.

Analysis of recovered livers revealed a reduction of liver fibrosis with thinner collagen septa after injection of ADHLSCs, as demonstrated by Sirius Red staining (Figure 2).

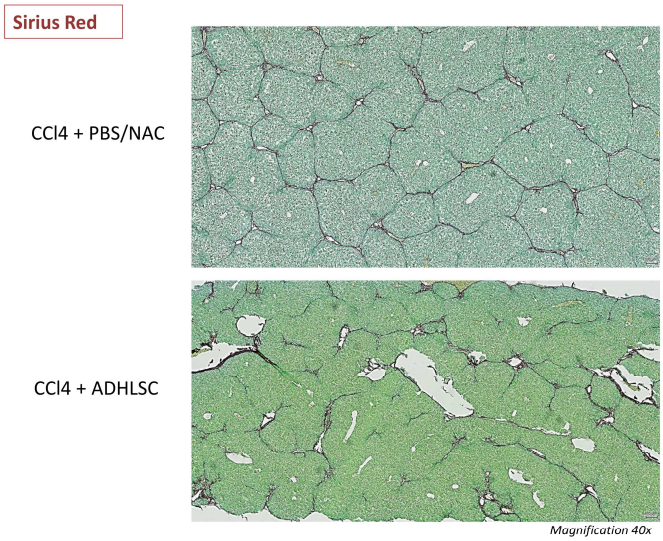

Figure 2: Sirius red staining in livers of SCID-Beige mice treated with CCl4 and intrasplenically injected either with the vehicle (PBS/NAC) or with ADHLSC. Magnification 40x.

In serial slices, we demonstrated that ASMA immunostaining was lower in the group transplanted with ADHLSCs, attesting to the presence of less activated HSCs (Figure 3), which is in correlation with the previous results. The decrease of ASMA was confirmed at the gene expression level, as illustrated in figure 4.

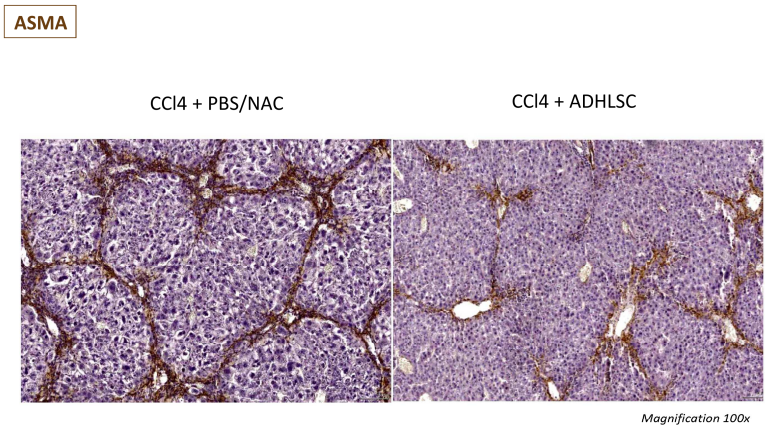

Figure 3: ASMA immunostaining in livers of SCID-Beige mice treated with CCl4 and intrasplenically injected either with the vehicle (PBS/NAC) or with ADHLSC. Magnification 100x.

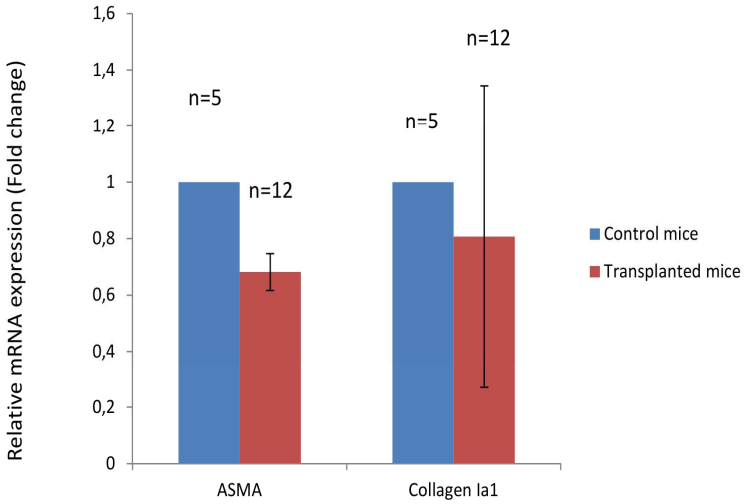

Figure 4: Gene expression of ASMA and Collagen Ia1 in the liver of SCID Beige mice treated with CCl4 and intrasplenically injected either with the vehicle (Ctrl) or with ADHLSC.

The analysis of the mice liver function revealed a tendency of a decrease in the level of ALT in the transplanted mice as compared to the control group (figure 5), which suggests an improvement of liver

function after cell transplantation. Two mice in the transplanted group have even reached the normal range.

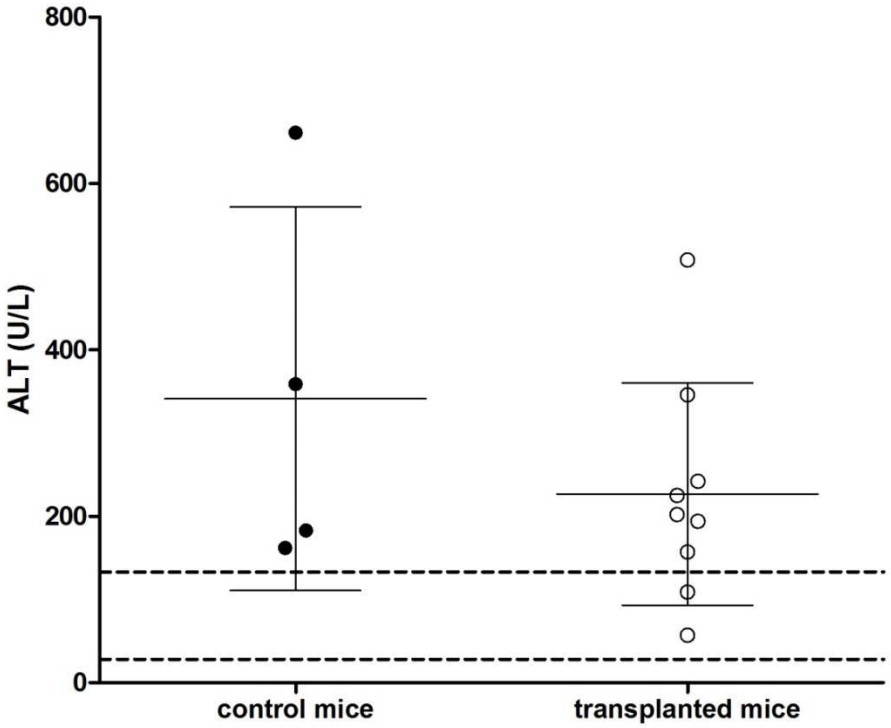

Figure 5: ALT levels in the serum of SCID Beige mice treated with CCl4 and intrasplenically injected either with the vehicle (Control mice) or with ADHLSC (transplanted mice).

Even preliminary, these results remain promising. Further studies will

include a larger number of animals as well as cells of different donors to get a clear cut information on the potential of ADHLSC to modulate the degree of liver fibrosis. The underlying mechanisms will also be studied and the effect of the secretome will be evaluated in vivo.

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Part IV

General discussion, perspectives and conclusion

General discussion

Liver fibrosis represents the consequence of a sustained wound healing process in response to chronic injury. The causes are numerous, including viral, autoimmune, drug induced, cholestatic and metabolic. Cirrhosis represents the end stage evolution of liver fibrosis, resulting in nodular transformation and loss of normal parenchymal architecture, impaired vascular flow and progressive impairment of the hepatic function. With hundreds of millions patients affected worldwide, cirrhosis represents a major public health issue [1].

The most effective treatment available nowadays remains orthotopic liver transplantation (OLT). However, organ shortage, operative risks, post-transplant complications and recurrence of the pre-existing liver disease underline the necessity to develop alternative therapeutic strategies [2]. Moreover, fibrosis can develop into the liver grafts. Transplant-related factors such as cold ischemia time and biliary complications are known to play a role in this phenomenon [3] [4].

Acting earlier on the progressive fibrous tissue deposition may prevent this ultimate stage.

It is now widely acknowledged that liver fibrosis is a dynamic and potentially reversible process [5]. A better understanding of liver fibrosis physiopathology is useful to define the strategic points of an optimal therapeutic intervention. Several strategies are currently focusing on reducing inflammation, protecting host hepatocytes, inhibiting HSC activation, stimulating activated HSC apoptosis, or directly modulating the extracellular matrix and improving its degradation.

Several drugs have been developed in order to meet these targets and their use has been reported in several preclinical and clinical studies. Although some preclinical studies have shown promising results, the efficacy of such therapies has not been proven in humans [6] [1] [7]. Differences in pharmacokinetics and in collagen cross linking between rodents and humans can probably explain these contradictory results. Moreover, most clinical studies are performed during a limited period of time while fibrosis takes years to develop. A longer follow-up period is probably needed in order to establish the efficacy of the drugs.

Over the past few years, cell-based therapy has progressively emerged as a potential promising alternative to OLT for patients suffering from metabolic disorders [8]. This less invasive technique has been proposed to treat liver fibrosis because of the ability of stem cells to improve the hepatic inflammatory microenvironment, inhibit the activation or induce apoptosis of HSC, replace damaged hepatocytes and promote the regeneration of residual hepatocytes [9]. Cell-based therapy presents the advantage to meet several targets and to have a durable action following engraftment in the liver parenchyma. Mesenchymal stem cells (MSCs) seem to be a promising candidate, because of their safer profile as compared to embryonic stem cells and induced pluripotent stem cells [10]. In vivo studies using MSCs to treat induced liver fibrosis have shown promising results [11] [12] [13]. A decrease of liver fibrosis is accompanied by a frequent improvement of the liver functions. However, there is no consensus regarding the exact mechanisms by which MSCs exert their beneficial effect and the interactions between MSCs and activated HSCs are not evaluated in such models. In human, the injection of MSCs has been followed by the improvement of the liver function in several clinical studies [14] [15], even among patients presenting an advanced stage of liver fibrosis. The MSCs used in the

different studies are of extra-hepatic origin (bone marrow, adipose tissue, umbilical cord). The beneficial effects were observed regardless of the origin of MSCs, even if the superiority in terms of immunomodulation has been demonstrated in vitro for adipose tissue-derived MSCs in comparison with bone marrow-derived MSCs [16].

In the current work, we investigated the anti-fibrotic properties of mesenchymal stem/ progenitor cells derived from the adult healthy human liver (ADHLSCs). These cells display characteristics described to be potentially associated to anti-fibrotic effects, such as the immunoregulatory properties and the differentiation potential, as described for extra-hepatic MSCs [17] [18]. However, in contrary with extra-hepatic MSCs, ADHLSCs have a predetermination to differentiate into hepatocyte-like cells both in vitro and in vivo and have the potential to participate to liver regeneration [18] [19] [20].

As HSCs represent the main cell type implicated in the extracellular matrix production, we investigated the ability of ADHLSCs to modulate HSC activation.

Even if HSCs are derived from the liver non-parenchymal fraction and ADHLSCs from the parenchymal fraction, we noticed a similarity in cell

morphology and proliferation potential. Moreover, numerous markers were expressed in both cell types, making it difficult to differentiate the two cell populations using the classical techniques used to characterize MSCs. Therefore, we extensively compared both cell populations, in order to propose tools allowing their discrimination. The comparative studies were performed on both cell populations isolated from the same donor.

The data of the current study revealed that HSC and ADHLSC are two different cell populations characterized by distinct gene expression and secretion profiles [21].

Thanks to this part of the work, we demonstrated a high expression level of HGF by ADHLSCs, a growth factor known to display anti-fibrotic properties [22] [23], like inhibiting TGFb pathway and inducing metalloproteinases expression and activity [24], as well as up-regulating miR-29 expression and thereby regulating collagen expression/secretion [25].

ADHLSCs also expressed higher levels of some metalloproteinases, such as MMP3, MMP2 and MM14, known to have anti-fibrotic properties as they are able to degrade several components of the extracellular matrix [26].

In parallel, we performed a functional analysis of ADHLSCs and HSCs by analyzing their secretion profile in basal conditions. These experiments allowed us to 1) confirm the singularity of each cell type and 2) support the hypothesis that ADHLSCs could potentially have anti-fibrotic properties, as they secrete cytokines of therapeutic and immuno-modulatory importance.

Indeed, we demonstrated that ADHLSCs highly secreted HGF, as well as anti-inflammatory cytokines (IL1-ra, IL-4, IL-10 and IL-13) and interferon gamma, an inhibitor of collagen deposition and HSC activation [27].

These results may suggest an anti-fibrotic potential of ADHLSCs, as it has been shown for other types of MSCs [28] [11].

In order to confirm the hypothesis that ADHLSC could have anti-fibrotic properties, we evaluated their ability to modulate the features

displayed by HSC after in vitro activation process. Indirect co-culture systems were used to answer this question. The model was developed because paracrine mechanisms have mainly been described to be implicated in the interactions between extra-hepatic MSCs and HSCs [29] [30] [31]. Moreover, the activation process of HSCs can be mimicked in vitro, as HSCs are in contact with the plastic dishes [32].

We demonstrated that autologous and allogeneic ADHLSCs were able to inhibit HSC proliferation, a major feature of HSC activation process. Soluble factors constitutively secreted by ADHLSCs seem to be implicated as the observed effects could also be obtained using ADHLSC conditioned medium.

The potent effect was observed with different ADHLSC/ HSC ratios (1/100, 1/10 and 1/1), but the effect was more pronounced with a ratio of 1 ADHLSC for 100 HSCs. We can make the assumption that an inhibitory molecule may be highly secreted by HSCs at high ADHLSC/ HSC ratios or that the implicated soluble factor(s) exert its action more efficiently at a determined concentration. In other published in vitro studies, extra-hepatic MSCs inhibit HSC proliferation in a dose-dependent manner [30] [29]. These differences may result from the fact

that extra-hepatic human MSCs and animal HSCs were used in these published studies while MSCs derived from the human liver and human HSCs were used in the current work. Species differences have been reported for HSCs [33]. Desmin is expressed in quiescent rat HSCs but not in quiescent human HSCs. ASMA is expressed in quiescent human HSCs but only in activated rat HSCs. Differences in receptor expression also underline the interest to use human HSCs in preclinical studies [34]. The fact that only a small number of ADHLSCs is needed to exert the therapeutic effect and the fact that allogeneic cells can be used seem attractive in a context of cell-based therapy.

Beside HSC proliferation inhibition, we also demonstrated that ADHLSCs were able to inhibit HSC pro-collagen type I secretion, after 24 hours of co-culture. As collagen type I represents the major component of the extracellular matrix, inhibition of collagen type I secretion represents an interesting therapeutic option to control liver fibrosis development.

In addition to previous studies, we also demonstrated that ADHLSCs were able to induce the production of anti-fibrotic molecules (such as

HGF, IL-6, MMP1 and MMP2) by HSCs themselves, suggesting the co-implication of an autocrine loop of HSCs in the effects observed after co-culture.

Even if MMP2 has been shown to be implicated in extracellular matrix turnover during tissue repair process (including degradation of collagen IV, laminin and fibronectin), its expression is increased during fibrogenesis [35] [36]. HSCs produce this enzyme during liver injury and both human and rat HSCs increase their expression of MMP2 during their activation process in vitro [36]. In the current study, we observed an increase in the secretion level of MMP2 by HSCs together with an inhibition of HSC activation process. These results may be explained by an alteration in the MMP/TIMP expression ratio, known to be critical in determining matrix remodelling, as it has been demonstrated for N-Myc downstream-regulated gene 2 (NDRG2) [37] [38]. In this study, NDRG2 induced an increase in the ratio MMP2 to TIMP2 and was able to inhibit HSC activation and to ameliorate liver fibrosis [37]. Further studies evaluating the secretion level of TIMPs by HSCs after co-culture with ADHLSCs and the activity of MMPs would be of great interest to understand the underlying mechanisms.

In order to investigate the mechanisms by which ADHLSCs could exert their effect on HSCs, we blocked the secretion of HGF, a growth factor highly secreted by ADHLSCs, as previously discussed.

HGF neutralization resulted in a partial reversion of HSC proliferation and pro-collagen secretion inhibition, suggesting that this growth factor is implicated (at least in part) in the observed effect on HSCs.

These results are in accordance with the previous data describing the ability of HGF to inhibit the PDGF-mediated over-proliferation of myofibroblasts in rat kidneys and to inhibit the pro-fibrogenic TGF-β pathway [24].

We demonstrated that the induction of anti-fibrotic molecules secretion by HSCs is also partially mediated by HGF. HGF is known to be able to induce MMP secretion by lung myofibroblasts [24]. If MMP1 and 2 are implicated in extracellular matrix degradation, IL-6 is involved in regeneration and survival of hepatocytes and can act synergistically with MSCs to reduce fibrosis in mice [39] [40].

A larger screening of ADHLSC secretome would be useful to identify the other factor(s) possibly implicated in the observed effects.

Perspectives

There is a growing evidence that MSCs can exert their therapeutic effects via the secretion of bioactive factors [41]. This is supported by i) the demonstrated ability of MSCs to exert therapeutic effects without a significant incidence or duration of engraftment in the transplanted animals and ii) the potential of MSC-conditioned medium to exert therapeutic effects, such as promoting liver regeneration in an animal model of acute liver injury [41] [42]. The paracrine activity of MSCs together with the modulation of inflammation, probably create a microenvironment favorable for tissue regeneration.

Beside cytokines and growth factors, MSC secretome also contains extracellular vesicles, including exosomes and microvesicles. Exosomes (50-100 nm in diameter) are vesicles generated by exocytosis of multivesicular bodies, while microvesicles (100-1000 nm in diameter) are generated by budding of the plasma membrane [41]. These extracellular vesicles contain proteins, as well as microRNA and RNA. In the context of liver fibrosis, transplantation of exosomes derived from human umbilical cord MSC was shown to alleviate CCl4-induced liver

fibrosis by inhibiting epithelial to mesenchymal transition (EMT) and by protecting hepatocytes [43].

The implication of extracellular vesicles in the paracrine modulation of HSC by ADHLSC is worth to be explored in the future, in parallel with a large screening of cytokines and growth factors contained in ADHLSC conditioned medium.

The in vitro model allows the study of the mechanisms involved in the interactions between ADHLSCs and HSCs. However, it presents some limitations as the whole organ is not intact. The influence of the different systems present in a living organism (nervous, immune, endocrine, circulatory,…) is not evaluated in vitro.

Such questions can be addressed in vivo. We have shown promising preliminary results of an in vivo study which evaluated the influence of ADHLSCs in an animal model of induced liver fibrosis. We observed a decrease of liver fibrosis in the group of mice transplanted with ADHLSCs. A second study is currently ongoing to confirm the data with a higher number of animals.

Moreover, the study of the mechanisms involved would be of great interest and might deserve a new PhD thesis work. Several hypotheses can be made, including 1) the differentiation of ADHLSC into hepatocyte-like cells, 2) the promotion of liver regeneration, 3) the secretion of MMP, 4) the improvement of the inflammatory microenvironment, 5) the modulation of HSC activation.

General conclusion

This work takes place in the context of MSC-based therapy development for the treatment of liver fibrosis. A first comparative study allowed us to point out several characteristics of ADHLSCs (including their secretion profile), that could suggest their potential anti-fibrotic properties. In vitro studies confirmed this hypothesis and revealed the ability of ADHLSCs to modulate the features displayed by HSCs after their in vitro activation. Beside the inhibition of HSC proliferation and collagen secretion, ADHLSCs were also able to induce the secretion of anti-fibrotic molecules by HSCs themselves, suggesting a possible co-implication of an autocrine loop of HSCs in the anti-fibrotic process induced by ADHLSCs. Our study highlights the role of HGF as a part of the potential mechanism involved. These results provide new insights in the mechanisms underlying the anti-fibrotic effects of MSCs, as HSCs themselves could be implicated in the therapeutic process. Additional in vitro and in vivo studies should support the potential development of ADHLSC cell-based systems in the treatment of liver fibrosis.

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List of publications

Berardis Silvia, Lombard Catherine, Evraerts Jonathan, El Taghdouini Adil, Rosseels Valérie, Sancho-Bru Pau, Lozano Juan Jose, van Grunsven Leo, Sokal Etienne Marc, Najimi Mustapha. Gene expression profiling and secretome analysis differentiate Adult-Derived Human Liver Stem/progenitor Cells and human hepatic stellate cells**.** PLoS One 2014;9:e86137.

Berardis Silvia, Sokal Etienne Marc. Pediatric non-alcoholic fatty liver disease : an increasing public health issue. Eur J Pediatr 2014, 173: 131-139.

Berardis Silvia, Evraerts Jonathan, El Taghdouini Adil, Henriet Patrick, Smets

Françoise, van Grunsven Leo, Najimi Mustapha, Sokal Etienne Marc. Human liver mesenchymal cells inhibit human hepatic stellate cell activation and induce their secretion of anti-fibrotic molecules via HGF mediated paracrine effect. Submitted.

Berardis Silvia, Dwisthi Sattwika Prenali, Najimi Mustapha, Sokal Etienne Marc. The use of mesenchymal stem cells to treat liver fibrosis: current situation and future prospects. In press.

Maerckx Cédric, Tondreau Tatiana, **Berardis Silvia**, van Pelt Jos, Najimi Mustapha, Sokal Etienne Marc. Human Liver stem/progenitor cells decrease serum bilirubin in hyperbilirubinemic Gunn rat. World Journal of Gastroenterology 2014. In press.

Communications

- **Congrès de la Société belge de pédiatrie**, Anvers, Belgium, March 15-16, 2013**.**

Poster presentation. Non-alcoholic fatty liver disease in children: an increasing public health issue.

- **Groupe cellules souches**, Brussels, Belgium, May 2nd, 2013.

Oral presentation. Evaluation des propriétés anti-fibrotiques des cellules souches/progénitrices issues du foie humain adulte.

- **12th Congress of the Cell Transplant Society**, Milan, Italy, July 8th, 2013.

Oral presentation. Gene expression profiling and secretome analysis differentiate ADHLSC and HSC.

- **IREC PhD Day**, Brussels, Belgium, September 26th, 2013.

Oral presentation. Evaluation of the anti-fibrotic properties of ADHLSC.

- **United European Gastroenterology Week (UEGW)**, Berlin, Germany, October 14th, 2013.

Poster presentation. Gene expression profiling and secretome analysis differentiate ADHLSC and HSC.

- **13th Asian Pan-Pacific Society for Pediatric Gastroenterology, Hepatology and Nutrition (APPSPGHAN) and 40th Japanese**

Society for Pediatric Gastroenterology, Hepatology and Nutrition (JSPGHAN), Tokyo, Japan, November 3rd 2013.

Oral presentation. Gene expression profiling and secretome analysis differentiate ADHLSC and HSC.

- **47 th Annual Meeting of the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPGHAN)**, Jerusalem, Israël, June 12th 2014.

Oral presentation. Evaluation of the in vitro anti-fibrotic properties of ADHLSC.