PROJECT FINAL REPORT

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1. FINAL PUBLISHABLE SUMMARY REPORT

1.1 EXECUTIVE SUMMARY

Chronic liver disease is the fifth cause of death in Europe. Due to the limited availability of donor livers, many people die while on the waiting list for a liver transplant, currently the only effective treatment for life-threatening liver diseases. However, this is not an ideal therapy since post-surgery complications and immunosuppressive drugs pose substantial risks for patients. Thus, development of safer, cost-effective methods for treating chronic liver disease reflects a health priority. The consortium LIVES has pursued the project "*Development of culture conditions for the differentiation of hES cells to hepatocytes*" to facilitate cell-based therapy for patients who require regeneration of liver function.

Clinical trials of hepatic cells isolated from donor livers have provided convincing evidence that transplantation of these cells can restore liver function, thereby suggesting this approach as a viable alternative to organ transplantation. However, to implement cell-therapy for chronic liver disease, a renewable source of functional human hepatic cells must be developed. Human embryonic stem cells (hESC) are able to grow indefinitely *in vitro* and differentiate into all cell types of the human body. Although hESC have been differentiated to hepatic cells by various laboratories, the current approaches are not suitable for clinical applications since they rely on animal-derived components and products with unspecific effects which pose risks to human health. The Liv-ES consortium was created to develop reproducible, animal-free conditions for the efficient differentiation of hESC to hepatic progenitors, with the aim of providing the European community with the necessary knowledge and tools to establish a renewable source of transplantable cells for treating chronic liver disease.

Liv-ES has made tremendous progress towards the development of novel reagents and methods for directing the differentiation of hESC to hepatic cells. One of our basic objectives was to create the technological tools to replace animal products in routine culture of hESC. The LIVES partners have generated biomaterials which support both the growth and differentiation of hESC and the bipotent cell line HepaRG. Moreover, some partners have developed a series of reporter cell lines which are invaluable tools for the identification and purification of cells expressing specific hepatic markers. Other partners have developed a step-wise, chemically-defined protocol for efficiently directing hESC to early-stage progenitors which are committed to the hepatic rather than pancreatic lineage. A renewable source of hepatic cells must expand in vitro: using the reporter tools to identify cells that express specific markers, Liv-ES partners have generated a method to purify hepatic progenitors which can then be expanded *in vitro*, an important step for future application to cell therapy. The pioneering technology of miRNA has also been employed during the project to identify novel markers of hepatic progenitors. When subjected to further differentiation, these hepatic progenitors yield functional hepatic cells which secrete albumin and express inducible cytochrome P450 3A4, the major detoxifying protein in adult liver, and are capable of engrafting in animal models. Finally, the HepaRG cell line has been a critical model for Liv-ES partners to further characterize the culture components and cellular mechanisms which regulate hepatogenesis.

Collectively, the results of Liv-ES provide solid groundwork for developing hepatocyte-based therapy as a standardized procedure of regenerative medicine. The findings of Liv-ES have been published widely in journals of high impact. Thus, the tools and methodology generated by this project are available to both basic and clinical scientists for translation to human liver diseases. The culture conditions developed and validated by Liv-ES will now enable new therapeutic strategies based on stem cells or engineered tissues for restoring liver function.

1.2 SUMMARY DESCRIPTION OF PROJECT CONTEXT AND OBJECTIVES

The field of regenerative medicine offers promising new possibilities for repairing or restoring the function of organs which have been damaged by disease or aging. Defective organs can be regenerated either through activation of endogenous repair mechanisms or by replacement, as in the case of transplants. Regenerative medicine using stem cells has attracted much attention, particularly because these cells are capable of auto-renewal and can differentiate into many types of cells. However, in order to develop regenerative medicine that is useful in a clinical setting, more adequate tools and methods for working with stem cells must be developed.

The liver is associated with many types of diseases, including metabolic disorders and acute liver failure. Chronic liver disease is the fifth cause of death in Europe. Once serious damage occurs to the liver, it loses the ability to repair itself and this is a life-threatening problem because the liver can no longer perform essential physiological functions including removal of toxins from the blood and the production of biochemicals for digestion. The only treatment currently available for chronic liver disorders is an orthotopic liver transplant (OLT). In Europe, inherited metabolic diseases represent 26% of the indications for OLT. However, the number of patients dying while on liver transplantation waiting lists has increased these last years as a result of the severe shortage of organs (11%), while it is as high as 80% in fulminant hepatic failure. There are also substantial mortality and morbidity risks related to post surgical complications (cell rejection, vascular and biliary obstructions), and immunosuppression (infections).

Liver-directed cell therapies, such as engraftment with allogeneic hepatocytes, offer alternatives to orthotopic liver transplantation for the treatment of metabolic disorders. During the last years, some 20 clinical trials have tested hepatocyte transplantation and the results have been encouraging as they have demonstrate that these cells can restore liver function in patients. However, these approaches suffer important limitations: in addition to the paucity in organ donors, adult hepatocytes can divide only once or twice, even in the presence of hepatocyte growth factor, and thus cannot be expanded in vitro. They are also difficult to cryopreserve and are highly susceptible to freeze-thaw damage, which reduces their functional capacity.

With regard to drug testing, the liver is central to pharmacokinetics and toxicology of xenobiotics, but animal models are often misleading because expression levels and substrate specificity of liver enzymes are different from their human counterparts. As a consequence, hepatic clearance and chemical profile of metabolites do not accurately represent human liver funtion. In fact, unexpected problems of toxicity and pharmacokinetics are responsible for 40-50 % of all failures in clinical drug development. Human cell systems, which include human hepatocyte cultures, immortalized cell lines and liver microsomes, have the potential to overcome these limitations but the cell models which are currently available are not amenable to toxicity testing for a variety of reasons. Expression of key liver enzymes, like CYP450, decline rapidly after hepatocyte isolation. Cell lines like HEP-G2 which are derived from tumors display very low expression of transporters and key liver enzymes (CYP450, conjugating enzymes), and do not exhibit the correct cell morphology and polarisation for vectorial drug transport from the plasma to the bile. These limitations for direct therapeutic applications and drug discovery emphasize the need to explore and develop new sources of human hepatic cells, which can be amplified *in vitro* and subsequently differentiated into functional hepatocytes.

Stem cells represent an ideal source for obtaining renewable cultures of hepatocytes for application to cell-therapy as well as drug testing. Adult livers contain two-types of stem cells that could be potentially exploited to generate hepatocytes:

(a) Mesenchymal stem cells are cells of extra-hepatic origin with potential therapeutic applications. However, recent reports suggest that these cells may not participate directing in liver regeneration but rather provide trophic support in injured livers by inhibiting endogenous hepatocyte death and stimulating their proliferation. Moreover, in culture, they enter replicative senescence after a limited number of population doubling.

(b) Liver progenitor and stem cells are present as “resting facultative stem cells” in a specific niche of the liver. They have been estimated to represent between 0.01% and 1% of liver

cells in newborn livers but, although they can be expended in vitro, do not display telomerase activity by contrast to fetal liver progenitor cells.

Human embryonic stem (hES) cells are perhaps the most viable option to make new hepatocytes in the laboratory for patients whose liver can no longer regenerate. The embryonic origin of these stem cells confers upon them two important properties: hESCs are able to grow indefinitely in vitro while maintaining pluripotency or the capacity to differentiate into all cell types including hepatocytes. The combination of these two properties suggests that hES cells hold great promise for future clinical application since they could be used to produce large quantities of differentiated cells for cell-based regenerative strategies. However, the generation of fully functional, human hepatocytes from hES cells under clinically-compatible conditions remains a major challenge. In the last few years, several groups have reported the differentiation of hES cells into hepatic cells using diverse culture systems. However, these approaches were all based on culture media containing serum, complex matrices such as matrigel, and/ or mouse embryonic fibroblasts as feeders. Animal components pose potential risks for human health and the use of these poorly defined reagents could complicate studies aimed at identifying the molecular mechanisms of human liver development. Moreover, the proliferative capacity of hESC must be carefully controlled for clinical applications since once transplanted, they have been shown in vivo to produce teratomas or adenocarcinomas. Clearly, there are still a number of fundamental questions that must be answered before stem cell-based therapies can be employed in the clinical setting.

Given the urgent need to develop alternative therapeutic strategies for the treatment of advanced liver disease, the consortium LIVES has pursued the project "*Development of culture conditions for the differentiation of hES cells to hepatocytes*" to facilitate cell-based regeneration of liver function.

The main objectives of this collaborative project were as follows:

I. Development of novel tools which will combine state-of-the-art materials science with cell biology to optimise culture conditions for the differentiation of hES cells: Innovative 3-D growth matrices, lentiviral vectors, Es-derived stage-specific cell lines, a miRNA platform devoted to ES-derived endoderm lineages and liver-specific endothelial cells will be developed to generate and expand pure populations of ES-derived hepatic progenitors (WP1) under animal-free conditions and define new markers.

II. Optimisation of Culture Conditions for the Generation of Anterior Definitive Endoderm multipotent stem cells: Defined, animal-free conditions will be developed on a reference hES cell line (H9) to efficiently drive differentiation to ADE stem cells; subsequently, proof-of-principle validation of these defined conditions will include testing the protocol with the hES cell lines derived by various Partners and *in vitro* and *in vivo* characterization (WP2).

III. Differentiation and expansion of hepatic progenitors from multipotent ADE cells using animal-free conditions: hepatic progenitors obtained by this protocol will be validated by comparison with foetal hepatic progenitors and the HepaRG hepatoma cell line and validated *in vivo* (WP3). This aim will perform *in vivo* evaluation of hepatic precursors to provide academic projects with fully characterized ES-derived hepatic progenitors (WP3).

IV. Differentiation of mature hepatocytes from ES-derived hepatic progenitors using animal-free conditions: Validation of novel hepatocyte lines for expression of detoxifying enzymes to provide the European pharmaceutical industry, SMEs and academic projects with efficient cells to allow drug testing. Products of this protocol will be carefully evaluated *in vivo* to ensure that these cells do not cause teratomas and that their metabolism and growth resemble functional, human heptatocytes, which will allow future preclinical evaluation for cell therapy (WP4).

1.3 MAIN S&T RESULTS

Human ES cells (hESC) hold great promise for future clinical application since theoretically they can be utilized to produce large quantities of differentiated cells *in vitro* for cell-based regenerative strategies. Patients with chronic human liver disease would benefit greatly from this advance since liver transplants are expensive and there are simply not enough organ donors to treat all the patients. However, the generation of fully functional, human hepatocytes from hES cells under clinically-compatible conditions remains a major challenge. In the last few years, several groups have reported the differentiation of hES cells into hepatic cells using diverse culture systems. However, these approaches were all based on culture media containing serum, complex matrices such as matrigel, and/ or mouse embryonic fibroblasts as feeders. Animal components pose potential safety issues for clinical applications and the use of these poorly defined reagents could complicate studies aimed at identifying the molecular mechanisms of human liver development. Moreover, the proliferative capacity of hESC must be carefully controlled for clinical applications since once transplanted, they have been shown in vivo to produce teratomas or adenocarcinomas. Obviously, there are still a number of fundamental questions that must be answered before cell-based therapies can be applied to treat liver disease.

To facilitate the development of hESC as a potential strategy for regenerating human liver, it is necessary to define the precise conditions for recapitulating liver development *in vitro*, including the molecular cues which promote the differentiation of stem cells to hepatocytes. The working hypothesis of LIVES has been that functional hepatocytes can be obtained by developing the appropriate culture conditions to mimic the sequential stages of development which occur in the human embryo, from pluripotent stem cells to endoderm cells, then bipotent hepatic progenitors to hepatocytes. For translation to clinical procedures, these conditions must be chemically defined to avoid the use of animal products, feeder cells, and chromatin modifiers. Thus, the principal objectives of the LIVES consortium have focused on the development of reagents and methods to standardize the culture and differentiation of hESC and bipotent cells to hepatic cells. The strategies for creating these tools were based on the need to purify, expand, and/or immortalize hepatic progenitors.

Workpackage 1 of the LIVES experimental plan has focused on the "Development of novel tools for generating ES-derived hepatocytes"

During liver development, hepatic progenitors are in contact with other types of cells, namely mesoderm and endothelial cells, and these interactions are crucial for inducing hepatic differentiation and specialization. Studies in mouse models have confirmed that endothelial cells are required for proliferation and maturation of ES-derived hepatic cells. Hepatic cells also interact strongly with proteins of extracellular matrix. In vitro three-dimensional, biomaterial-based matrices may provide the optimal microenvironment for ES cell differentiation. Importantly, these biomaterials for cell culture can be prepared using animal-free materials and obviate the need for feeder cells, thereby enabling the development of much needed GMP-level protocols. Biomaterials are easily expanded to larger scale 3-D cell cultures in bioreactors.

Third-generation vectors derived from lentiviruses to express fluorescent marker proteins [eGFP (green fluorescent protein), RFP (red fluorescent protein)] under the control of different hepatic promoters appear efficient tools to identify and purify differentiating cells and to generate stable reporter cell lines. These cell lines will be useful to monitor the differentiation of ES cells under different conditions. Lentiviral derived vectors carrying immortalizing genes should permit to establish cell lines cells at different stages of differentiation.

Micro-transcriptome RNAs (miRNAs) are endogenous ~22-nucleotide non-coding RNAs known to regulate gene expression at both the translational and transcriptional levels. Many miRNAs are differentially expressed during the course of development and differentiation, and are thought to play important roles in these processes. Thus miRNA profiling appears to be a promising tool that may be used to monitor ES cell differentiation into endodermal cells, hepatic progenitors then

hepatocytes and compare the profiles with that obtained with HepaRG cell line at the stage of progenitors and hepatocytes.

The effect of various ECM-proteins on hepatic differentiation have been tested. Various 3D commercial biomaterials have been also evaluated in terms of biocompatibility using 3D imaging and cellular viability tests, as well as functionality.

New biomaterials based on different components such as copolymers of hyaluronic acid and polyethyleneglycol were synthesized. The different materials evaluated had different cross-linking densities, porosity and chemical composition.

Evaluation of 3D imaging methods have been set up using first HepG2 cells to study expression and localisation of key proteins of polarized hepatocytes namely efflux proteins (MDR1, MRP, BCRP and BSEP).

After extensive comparative studies on HepaRG cells and ES-derived ADE cells, finally a commercial biomaterial (Hydromatrix), and a new biomaterial Nanocellulose, which has been developed in P6 lab were selected for further development to optimize the 3D culture condition used for differentiation of hES cell-derived hepatic progenitors into hepatocytes (see below).

Lentiviral based vectors with stage-specific promoters (provided by different partners) driving the expression of two fluorophores: eGFP and dsRED were constructed and produced (P5). The expression and the tissue specificity of these lentivectors, once validated by the different partners, were then provided as highly concentrated and purified batches. Lentivectors with immortalizing genes have been also provided. Highly concentrated batches of non-integrative lentiviral based vectors were produced to allow for selection of hepatic progenitors based on specific reporter expression. Finally, EF1-puro-IRES silencing constructs to stably knockdown IRS2 in hES cells have been validated in HepaRG cells. Another version of most of the vectors was constructed in which a gene for selection (neomycine) was inserted and provided to partners.

Summary of Viral vector tools generated and tested by LIV-ES

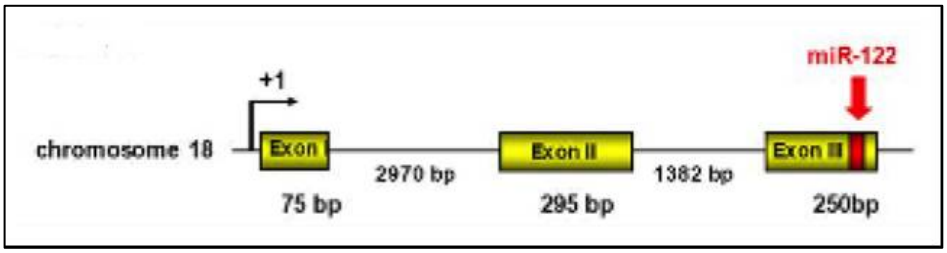
Lentiviral Vector			Target	Reporter Activity - Cell lines tested								
Promoter	GFP	dsRED	Cell	293T	COP7	Min6	HepG2	HepaRG precursor	HepaRG Hepato	Fetal Endo	hESCs Undiff	hESCs Diff
AAT	x		Hepatocyte	n	n	-	-	y (low)	y (low)	-	-	-
Alx3	x		pancreas	n	n	-	y (low)	n	n	-	-	-
ApoA2	x		Hepatocyte	n	n	-	y	y (low)	y (high)	-	n	?
ApoA2		x	Hepatocyte	-	n	-	-	y (low)	y (high)	-	-	-
CDK1		x	varios	-	-	-	-	y	y	-	-	-
Cyp3A4	x		Hepatocyte	-	-	-	-	y (low)	y (high)	-	-	?
Insulin	x		beta cell	n	y	y	n	-	-	-	n	n
Insulin		x	beta cell	n	n	-	n	-	-	-	-	-
IRS2	x		Hepatoblast	y	y	y	y (high)	y (high)	y (low)	-	n	y
miR122	x		Hepatocyte	-	-	-	-	y (low)	y (high)	-	-	-
miR122		x	Hepatocyte	-	-	-	-	y (low)	y (high)	-	-	-
PDX1	x		DE	n	n	-	n	n	n	-	-	?
EF1	x		All Cells	y	y	y	y	y	y	-	y	y
EF1	E6E7		All Cells	-	-	-	-	-	-	y	-	-
EF1	hTERT		All Cells	-	-	-	-	-	-	-	-	y

Efficient protocol to transduce pluripotent hES cells was defined and the protocol shared with partners. Stable ES cell lines transduced with EF1alpha-GFP have been generated as control cell line. Stable lentiviral reporter lines in hES cells have been also generated with APOA-II-GFP,

Insulin-GFP and IRS2-GFP using H9 cells as well as the Spanish VAL9 cells. The expression of both IRS2 and APOA-II were shown to coincide with the specification of hepatic progenitors. However, upon passaging APOA-II induction was not observed as well as that of Insulin, which suggest promoter specific silencing in undifferentiated ES cells.

Reporter HepaRG cell lines have also been established with the same APOA-II-GFP, and IRS2-GFP vectors. HepaRG cells were also transduced with the CYP3A4-GFP leentivector to analyse the influence of the microenvironment on the phenotype and the differentiation potential of HepaRG progenitors, to test the effect of DMSO and inducibility of GFP expression by rifampicin, a known inducer of CYP3A4 expression. Stable HepaRG cell line with IRS2 knockdown was also established to assess the differentiation and cell cycle markers (P4, P1C).

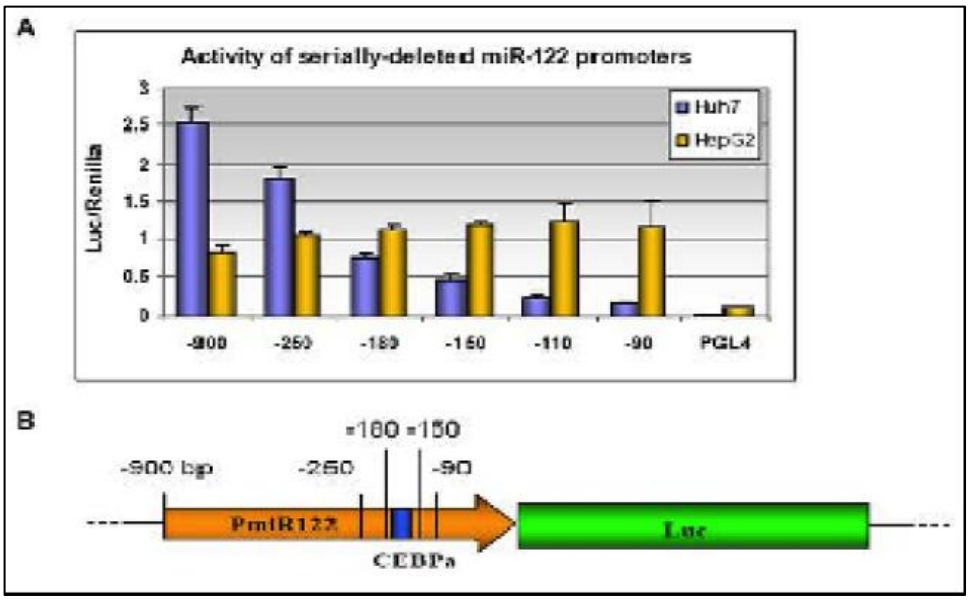
Many co-culture experiments performed between HepaRG and circulating progenitor endothelial cells and ES-derived endothelial cells failed to induce these cells to acquire a liver phenotype. Therefore conditions to isolate and purified foetal liver endothelial cells from mouse and human foetal livers have been set up. Immortalized cell lines have been generated and characterized (P1B).



Amongst the microRNA population, miRNA 122 has been identified as an essential and predominant microRNA expressed during liver development. This miRNA is liver specific and expressed at high level in the adult liver where it represents about 70% of all miRNAs. To better understand the role of this microRNA the molecular mechanism controlling its expression have been investigated. It was found that miR-122 primary transcript consists of 3 exons and that miR122 is generated from the 3rd exon.

Characterization of the miR-122 promoter showed that this promoter was expressed only in cells (human hepatoma cells) that express miR-122 and that there was a correlation between the level of expression of miR-122 and miR-122 promoter activity: Huh7 which express a high level of miR-122 have the highest promoter activity (compared to HepG2 and PLC/PRF5).

The miR-122 promoter was also found to have an enhancer activity for the liver specific human alpha1 antitrypsin promoter (hAAT) and not on control EF1alpha promoter.



Dissection of the promoter was made by serial deletions in front of the luciferase gene and it was shown that the site for the CCAAT/enhancer-binding protein-alpha (CEBP) was required for maximal activity.

Other results suggest that other factors such as HNF3B are required for optimal miR-122 promoter activity (P3). These results lead to the identification of

miR-122 promoter which was sent to Liv-ES partners in particular P5 for cloning in lentiviral vectors and use for monitoring hepatic progenitor differentiation toward more mature hepatocytes.

Workpackage 2 of LIVES focused on "Differentiation of hES cells into multipotent endoderm stem cells".

Generating functional, differentiated hepatocytes from hES cells using culture conditions that are compatible with clinical applications remains a major challenge. Development of a protocol which reproduces in vitro the early steps of human embryonic development may provide the best approach for generating differentiated cells with functional properties. The first event of differentiation during mammalian development occurs at the gastrulation stage with the specification of the primary germ layer ectoderm, endoderm and mesoderm from which all the cell types of the adult body are derived. The endoderm germ layer is quickly patterned during gastrulation to give rise to the anterior definitive endoderm (ADE) from which the hepatic bud will be later specified. Therefore, DE and then ADE cells represent the earliest progenitors of liver cells during development.

The effect of various ECM-proteins were tested on hepatocyte differentiation on hESC-derived DE cells such as Col 1, Col III, Col IV, fibronectin, gelatin and growth factor reduced matrigel. Based on immunostaining and Alb secretion it was concluded that fibronection may support ADE cell differentiation into hepatocytes and could be used for 3D-cultures.
For 3-D cultures various commercial matrixes were tested and amongst them, three that were chosen for more detailed studies: HydroMatrix (Sigma), Hystem-C (Sigma) and ExtraCel (TebiBio). Studies were performed on HepG2 cells then on HepaRG cells (P6A/B).

A protocol driving differentiation of hESCs into homogenous population of endoderm cells in chemically defined medium which is devoid of animal products and of source of undefined factor such as Matrigel or chromatin modifiers was developed (P2). These cells have been extensively characterized by expression of specific markers (Hex, CXCR4, Cerberus, Otx1, HNF1beta, goosecoid, MixL1, HNF3beta) by Q-RTPCR, immunostaining and micro-array gene expression profiling. Cells were also validated for the absence of expression of visceral endoderm markers. Importantly the protocol was found to be also effective on Finnish (FE22, 29) hES cells as well as on Spanish VAL9 hES cells.

For ADE cells to be considered as authentic endoderm progenitor, they must be competent to generate multiple endoderm cell types (liver, pancreatic cells and even lung).

Available methods to generate pancreatic progenitors often contain undefined animal products such as feeders or foetal bovine serum (FBS). Starting from our ADE cells, we screened defined culture conditions to differentiate human definitive endoderm (DE) into a near homogenous population of pancreatic and liver specified endoderm (see below) from multiple hPSC lines. The result of this screening shows that RA has an essential function in promoting pancreatic specification while BMP signalling blocks the expression of pancreatic markers. In addition, we observed that inhibition of FGF signalling decreases cells survival of pancreatic progenitors, thus justifying the use of FGFs in our protocol. More importantly, our analyses also revealed that Activin/TGFb controls DE cell fate choice toward the pancreas lineage by inhibiting dorsal foregut (DF) specification while promoting the hepatic lineage.

These results have important practical significance since protocols currently available to generate pancreatic cells from hPSCs often rely on feeders, Matrigel™ and serum all which represent potential source of TGFb signalling with the capacity to compromise pancreatic specification.

Finally, a chemically defined 2D platform was also developed for the generation of multipotent endoderm progenitors which can produce lung, liver and pancreatic progenitor cells. A methodology to expand this foregut endoderm population to produce homogeneous population of cells was also developped. All data has been replicated in multiple hESC and hIPSC lines demonstrating this is not a cell line or cell type specific observation (P2).

The third workpackage focused on the "Differentiation of endoderm stem cells into hepatic progenitors"

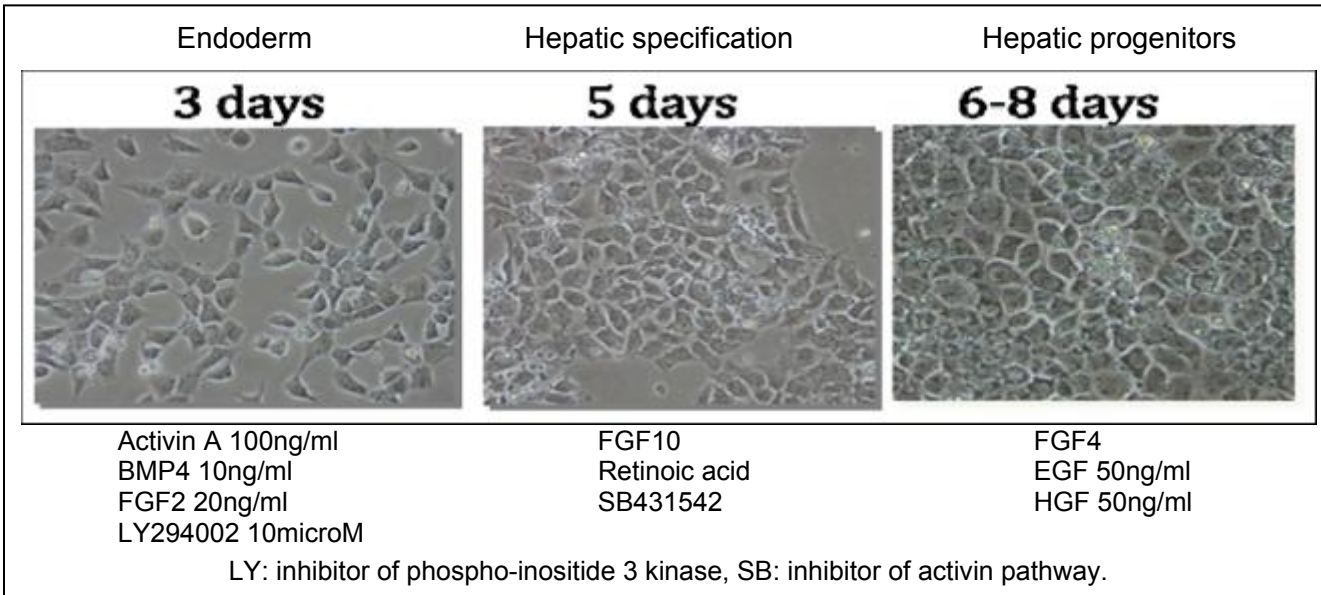
During embryogenesis, distinct mesodermal signals, either secreted or membrane-bound, are required for the specification of the liver bud and proliferation of foetal hepatic progenitors. The conditions developed to convert ADE cells to hepatic progenitors *in vitro* were based on our understanding of the processes that occur during normal liver organogenesis. The use of hepatic-specific primary endothelial cells was one critical approach as these cells are known to play a major role in promoting the proliferation of hepatic progenitors *in situ*. Human foetal hepatic progenitors (isolated and characterized as defined by Partner 1A) as well as HepaRG cells (developed and patented by Partner 1C) served as positive controls for the analysis of hepatic progenitors produced within this WP.

The inductive effect of various growth factors involved in liver bud organogenesis; such as FGFs, BMPs and inhibitors of signalling pathways (PI3Kinase) were evaluated on ADE cells generated from hESCs. The resulting culture system was devoid of animal products, serum or chromatin modifyers and was refined in order to achieve successful differentiation of ES-derived ADE cells to hepatic progenitors and fetal hepatocytes using fully defined culture conditions (P1A/P2).

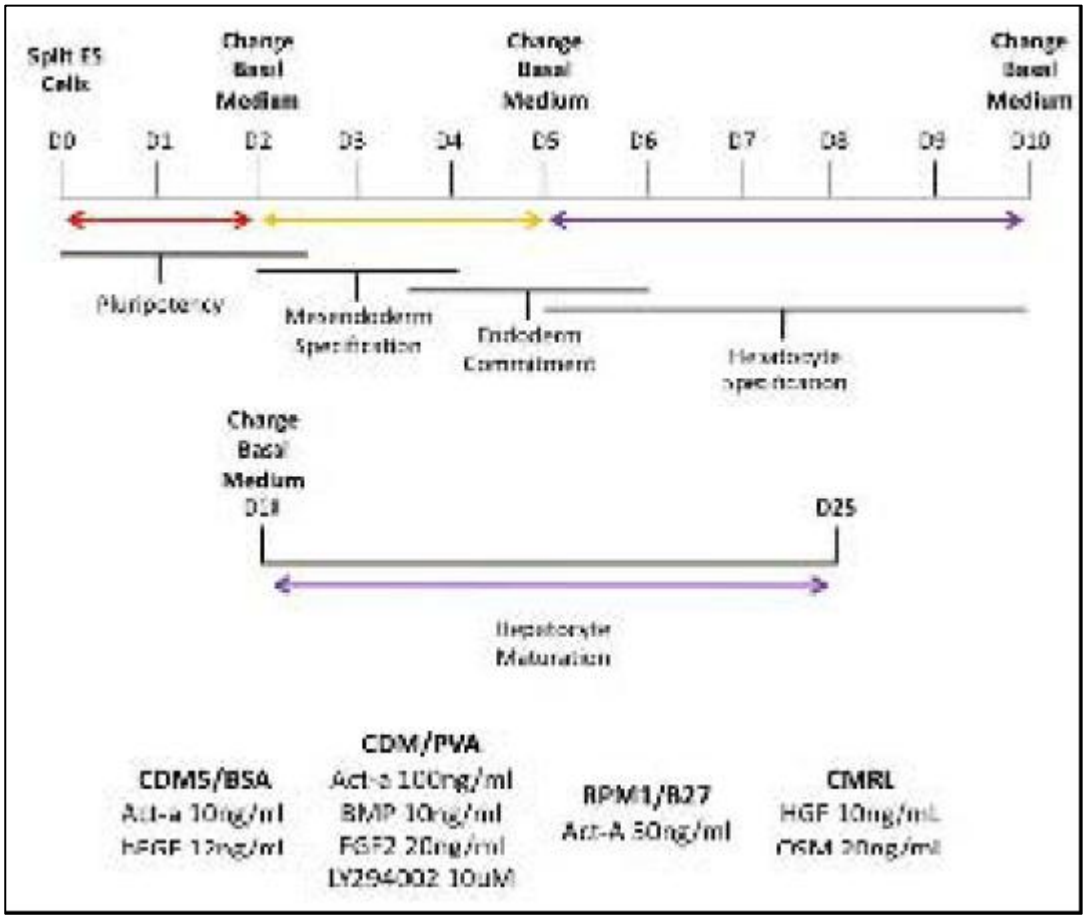
The ability of various cytokines to enhance differentiation towards endodermal lineages was analysed. These studies showed that TGFbeta/activin favoured hepatic specification. Therefore, a novel cell culture system was developed by P2 to generate fetal hepatocytes in which hepatic specification was obtained by replacing the CDM/PVA medium + factors by RPMI+B27+ Activin 50ng/ml. Further differentiation was then achieved by culturing the progenitors in CRML medium+HGF 10ng/ml +oncostatin 20ng/ml from day 10 to day 25 (P2).

Hepatic progenitors derived by both methods were characterized by Q-RT-PCR, RT-PCR, immunostaining and FACS analysis for cell surface markers. Functional studies were also used to characterize and validate the cells generated. These included; urea excretion assays, albumin synthesis, indocyanin green uptake and excretion and glycogen storage.

Summary of the first published protocol to generate hepatic progenitors. Cells were cultured in CDM/PVA medium.

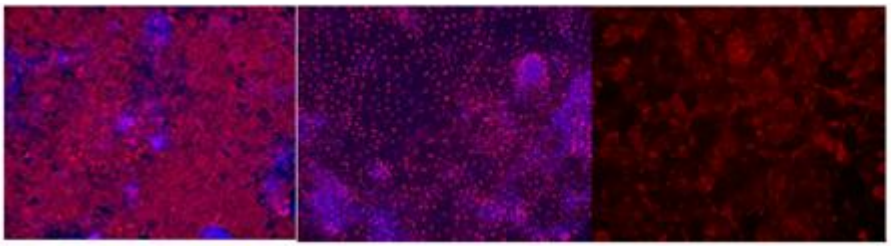


Summary of the second published protocol generated by P2.



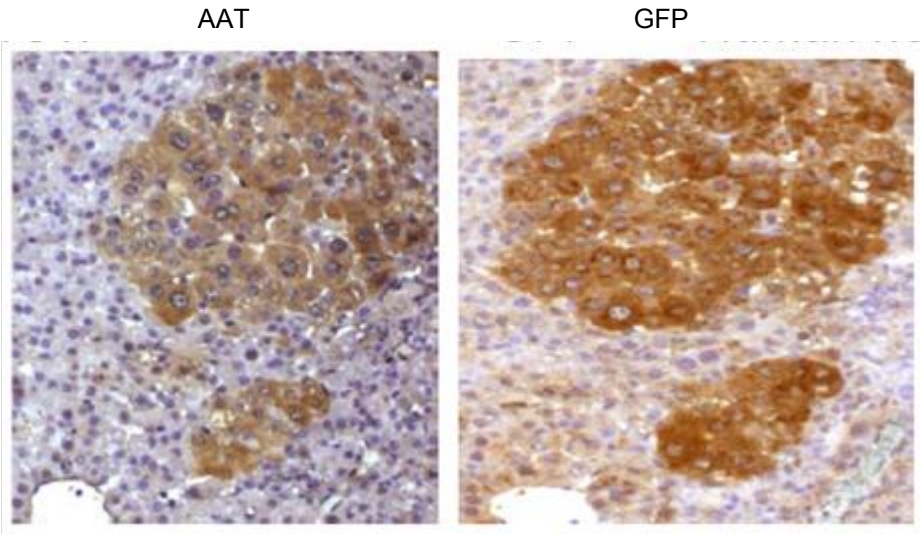
An example of hepatic markers expressed by hepatic progenitors expressed between d12 and D16 of differentiation. AFP and HNF4alpha are markers of the hepatocyte lineage whereas CK19 is a marker of cholangiocyte lineage.

When ES cells were transduced with APOA-II-GFP vector and differentiated into hepatic progenitors expression of GFP was observed in most cells.



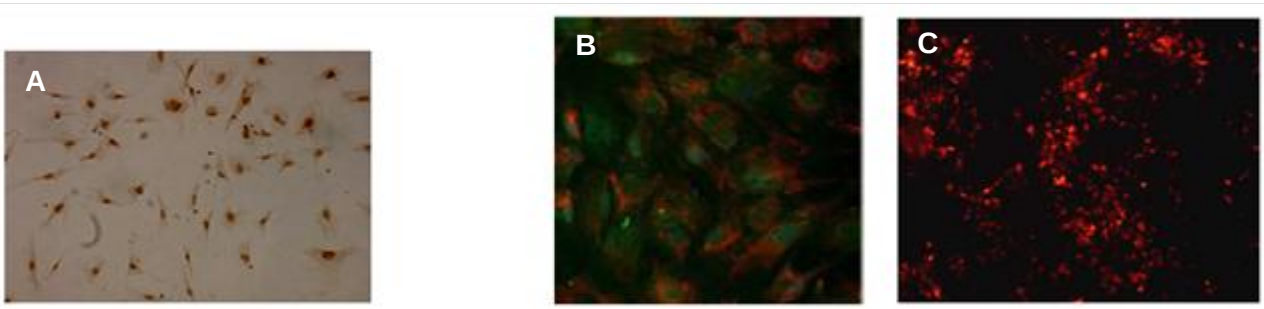
AFP HNF4alpha CK19 ApoA2 Expression

Importantly, the hepatic progenitor cells derived *in vitro* from hESCs using the first protocol, were able to graft and maintain their differentiated characteristics *in vivo* following transplantation into an immunodeficient (Rag2gammac-/-x uPa) mouse model. These studies demonstrated the presence of clusters of grafted cells expressing human hepatocyte markers AA1T and albumin within the liver parenchyma of the transplanted animals.



Human hepatic cells detected in transplanted mouse liver

Cell culture conditions were developed and defined during the study that would allow the passage and expansion of progenitor cells generated by the differentiation protocol. The hepatic progenitors derived from hESCs from days 13 to 15 of the protocol could be amplified on collagen 4 and Collagen I coated plates, in chemically define -hepatic progenitor culture medium (HPM). Amplification of hepatic progenitors also enabled us to define conditions to then purify these cells. The approach used was based on a combination of cell sorting, a method for producing purified integrating (ILV) and integration-defective lentivectors (IDLV) yielding high-titer pure preparations of viral particles (P5) and the use of a hepatic promoter and of a specific integrase inhibitor.
The human apolipoprotein A-II (APOA-II) promoter was used to drive GFP expression in hepatocyte progenitor cells following infection with either integrating lentivirus or with Integrase-deficient lentivirus. The specificity of APOA-II-GFP lentivirus was validated in various cell lines and in differentiating hES cells prior to being used to isolate pure cultures of progenitor cells from differentiating hESCs.
Conditions for cell sorting of the hepatic progenitor population yielded a population of 99% GFP positive cells 89% of which co-expressed hepatoblast markers such as AFP and CK19.

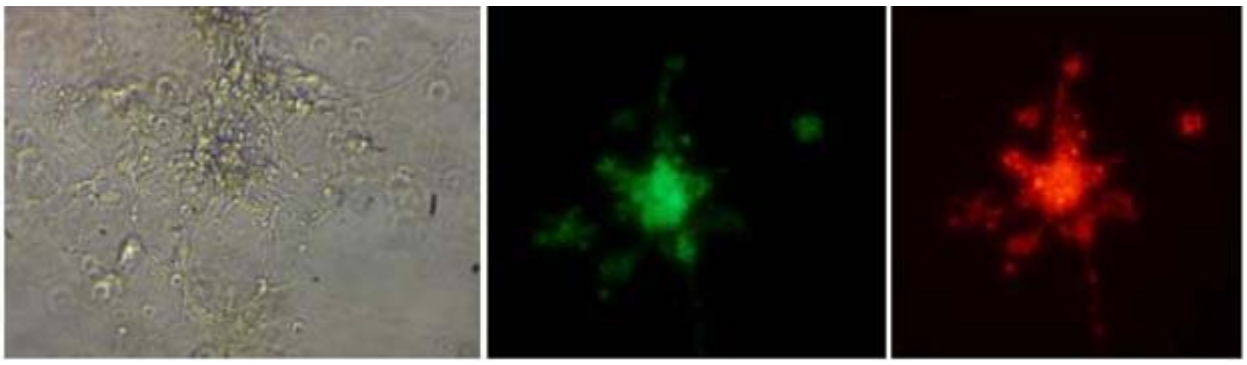


A - Purified proliferating progenitors after sorting stained with anti-PCNA
B - Representative field of purified progenitors co-expressing AFP (green) and CK19 (red)
C progenitors transduced with miR122-RFP

In order to circumvent the potential drawbacks of using integrating viral reporters to label precursor cells, one of the major objectives of the study was to develop an approach to temporarily label precursor cells with the APOA-II reporter so that they could be isolated without permanent genetic alteration. We therefore developed the use of Integrase-defective lentivirus and a viral integrase inhibitor (raltegravir) to express the selectable reporter in a fully reversible manner. Hepatocyte

precursor cells generated using these methods were shown to be integration free and could be differentiated further into more mature hepatocytes expressing albumin (P1A).

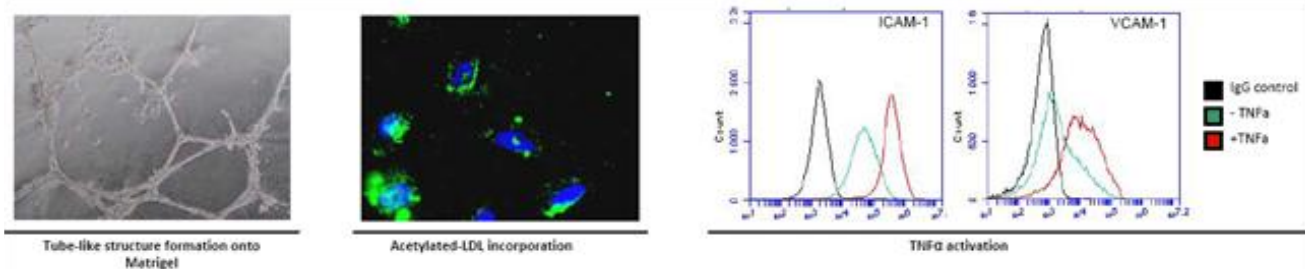
Whether proliferating hepatoblasts behaved as true bipotential progenitors and were able to also undergo differentiation to the cholangiocyte lineage has been investigated. Conditions have been defined to generate fetal cholangiocytes, co-expressing HNF6 and CK19 and which are able to adopt tubular forms, characteristic of cholangiocytes.



HNF6 CK19

HepaRG cells at the stage of progenitors were also shown to be able to differentiate into cholangiocyte like cells expressing specific markers such as GGT1 or KRT14 (P1A/C).

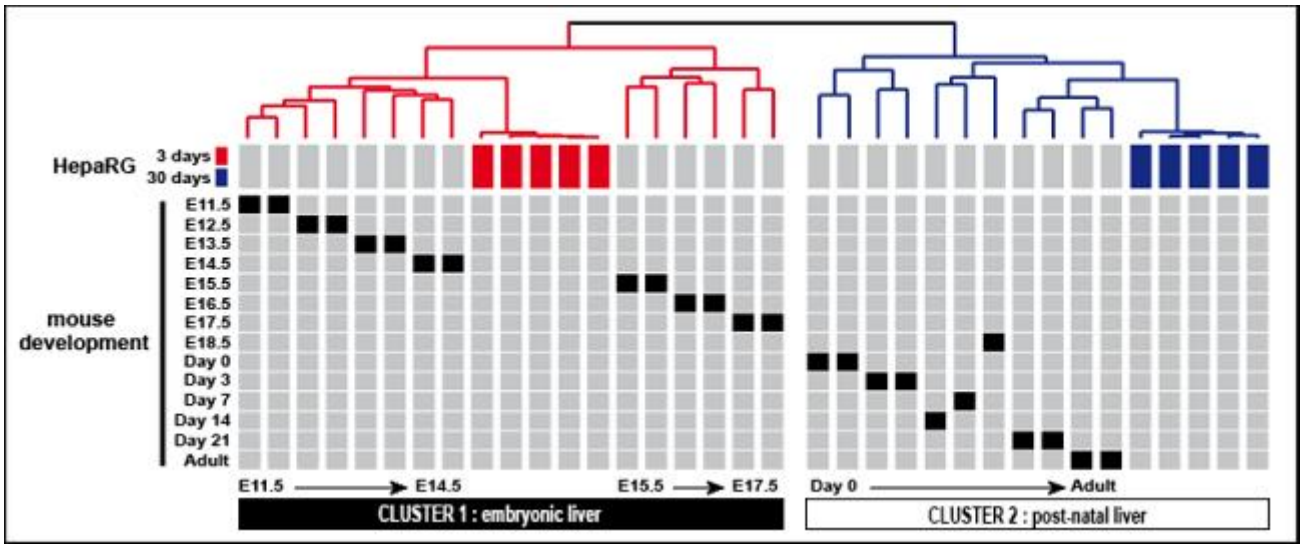
Endothelial cells isolated from dissociated liver were sorted according to expression of CD146 and CD31 and the resulting cells were demonstrated to be Lyve-1 positive (a marker of liver sinusoidal endothelial cells). Following isolation the cells were immortalized by lentiviral infection with EF1 driven hTert and E6-E7. Using this method different immortalized human liver endothelial cell lines were generated and characterized. All lines tested expressed endothelial markers (CD31, CD144, KDR, CD146) and were able to form tubules in matrigel, incorporate acetylated LDL and were activated by TNF-alpha (induction of ICAM-1 and VCAM-1). Coculture with hESC derived endodermal cells is a work in progress (P1B).



Specific functional tests on endothelial cells at passage 9

Little is known regarding the complex expression patterns of both mRNAs and miRNAs during the early stages of human liver development, and the role of miRNAs in the regulation of this process has not been studied. HepaRG cells were used because they reproduce the process of differentiation of bipotent hepatic progenitors and are capable of differentiating into both mature hepatocytes and biliary cells. Analyses were performed comparing the gene expression profiles of HepaRG progenitors at D3 and differentiated HepaRG at D30 without DMSO. They revealed profound changes between transcriptome of progenitors and differentiated HepaRG. More than 2000 genes whose expressions are changed more than 1.5 fold and 1500 whose expression are changed more than 2 fold between the two conditions. Interestingly, addition of DMSO between D15 and D30 known to potentialize the differentiation process induced also profound transcriptome

changes with a strong increase of several drug metabolizing enzymes expression, coagulation protein expression and a decrease of cell cycle protein and cell communication protein.
Comparison of the gene expression profiles of progenitors on Day 3 and differentiated HepaRG Day 30 without DMSO with gene expression profiles obtained from mouse livers at different stage of development was then performed. Hierarchical clustering analysis also allowed P1C to identify two major clusters which organization was driven by the culture condition of HepaRG cells. The first cluster corresponds to embryonic liver and includes HepaRG progenitors. The second cluster corresponding to post-natal liver includes differentiated HepaRG. Interestingly, the HepaRG signature in cluster 1 fit with early liver development stage corresponding to liver bud growth and liver hematopoiesis which demonstrates that HepaRG progenitor is a hepatoblast.



These transcriptomic profiles of HepaRG cells at the stage of progenitors and differentiated cells were then used to predict miRNA expression by using MiRABELLE software developed by P3. Three microRNAs (miR-210, miR-488, and miR-380-5p) were predicted to have a significant increased activity in progenitor HepaRG compared to differentiated HepaRG.

In parallel, another approach consisting to hybridize RNA from HepaRG on Agilent chip (G4471A Unrestricted Amadid miRNA 8x15k) was performed. Eight miRNA highly expressed in HepaRG progenitors compared to differentiated HepaRG (fold change > 2, $p<0.05$) were identified: **miR-17, miR19a, miR-221, miR-301a, miR-31, miR-34c-5p, miR-424 and miR-99p.**

Interestingly, among these miRs, miR-17 and 19a were shown to be expressed in liver at early stages of development (E11.5) and to decrease during liver organogenesis (*Jevnaker et al., J Cell Physiol. 2011;226:2257-66*). Using a mouse model of liver cancer, it was also shown that miR-221 overexpression stimulates growth of tumorigenic murine hepatic progenitor cells (P1C).

Global gene and miRNA expression profiled from adult and 9-12w human embryonic livers were obtained (high-density microarrays and quantitative RT-PCR) by P3. Comparison of the expression of the most regulated miRNAs to that of their putative targets using a novel algorithm revealed a significant anti-correlation for several miRNAs, and identified the most active miRNAs in embryonic and in adult liver. Furthermore, our algorithm facilitated the identification of TGFβ-R1 as a novel target gene of let-7.

miRNA expression profiled from undifferentiated and NaButyrate-induced differentiated hESC of two lines were also performed using microarray and quantitative RT-PCR. The miRNA profiling revealed expression of three novel miRNAs in undifferentiated and differentiated hESC. Upon NaButyrate induction, two of the most upregulated miRNAs common to both cell lines were miR-24 and miR-10a, which target genes shown to inhibit endodermal differentiation. In parallel, induction of several liver-enriched miRNAs, including miR-122 and miR-192, was observed to induction of endodermal gene expression. Moreover, ectopic expression of mir122 in hESC cells promotes

expression of HHEX and CXCR4, transcription factors required for generation of endoderm-derived progenitors. Therefore, mir122 was identified as a specific marker of the endoderm lineage that is also expressed early in liver development (P3).

It has been shown that insulin signalling via IRS2 is required for the proliferation and differentiation of HepaRG precursor cells. Knockdown of endogenous IRS2 prevented the cells from differentiating to hepatocytes, whilst omitting insulin from the differentiation media resulted in a blocked state of differentiation in which IRS2 was highly expressed. Insulin/IRS2 signalling was found to promote the proliferation of precursor cells and downregulation of endogenous IRS2 was necessary for proper maturation of HepaRG cells to hepatocytes.

Using pIRS2-GFP and APOA-II-GFP or dsRED lentivirus P4 made reporter cell lines and showed that distinct populations of cells in mature cultures expressed IRS2 or APOA-II, the latter corresponding to the more differentiated cells. All the data suggest that elevated expression of IRS2 marks a transitional state between precursor and mature hepatocyte and that insulin and IRS2 drive the early differentiation of bipotent progenitor cells. This hypothesis has recently been confirmed by double labelling of cells with pIRS2-GFP and APO-II-dsRED showing that APO-II-dsRED cells arise from IRS2 positive cells in the early stages of differentiation (P4).

The capacity of differentiation of hepatic progenitors toward the pancreatic pathway, was evaluated by growing these cells for 12 days in culture conditions inductive for pancreatic progenitors. Cells expressing PDX1/Sox9 could not be generated.

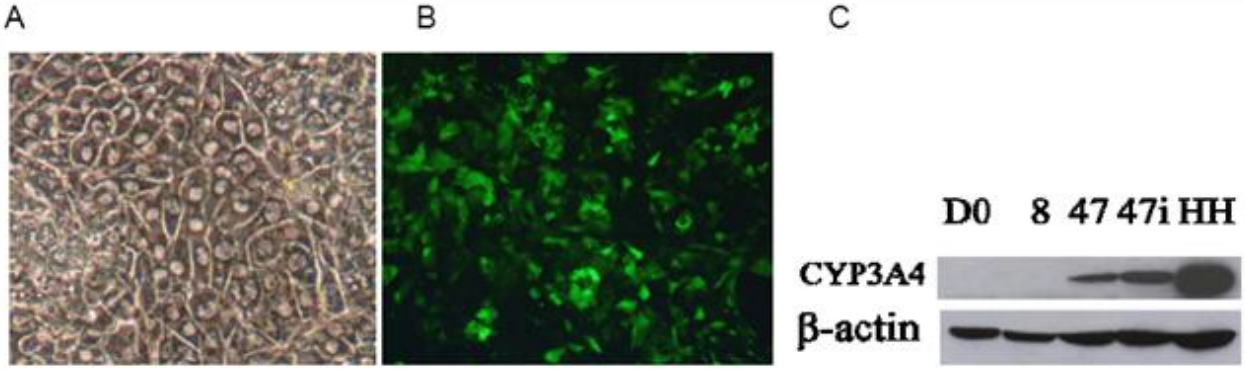
Use of H9 cells reporters for human insulin and Alx-3, markers which facilitate identification of pancreatic progenitor cells confirmed that hepatic progenitors were incapable of differentiating to cells of the pancreatic lineage.

These results from both P2 and P4 suggest that the differentiation protocol developed by the Liv-ES consortium efficiently induces specification of the hepatic lineage since our ADE-derived progenitors do not yield pancreatic cells when subjected to differentiation conditions that generate precursors of the pancreatic lineage. These hepatic progenitors have been fully characterized *in vitro* and validated *in vivo*.

The fourth workpackage focused on "Differentiation of hepatic progenitors into mature hepatocytes"

Animal-free culture conditions (CDM+factors) have been defined for further differentiation of the population of fetal hepatocytes into more mature hepatocytes. These cells have been characterized for hepatocyte functions (P1A).

Lentiviral vectors expressing GFP under the control of CYP3A4 were also used for hepatocyte characterization.



Protein CYP3A4 was revealed by Western blot as compared to human hepatocyes (HH)
A morphology of differentiated hepatocytes; **B** CYP3A4-GFP - expressing hepatocytes; **C** ES-derived hepatocytes were cultured for 45 days and induced or not by rifampicin.

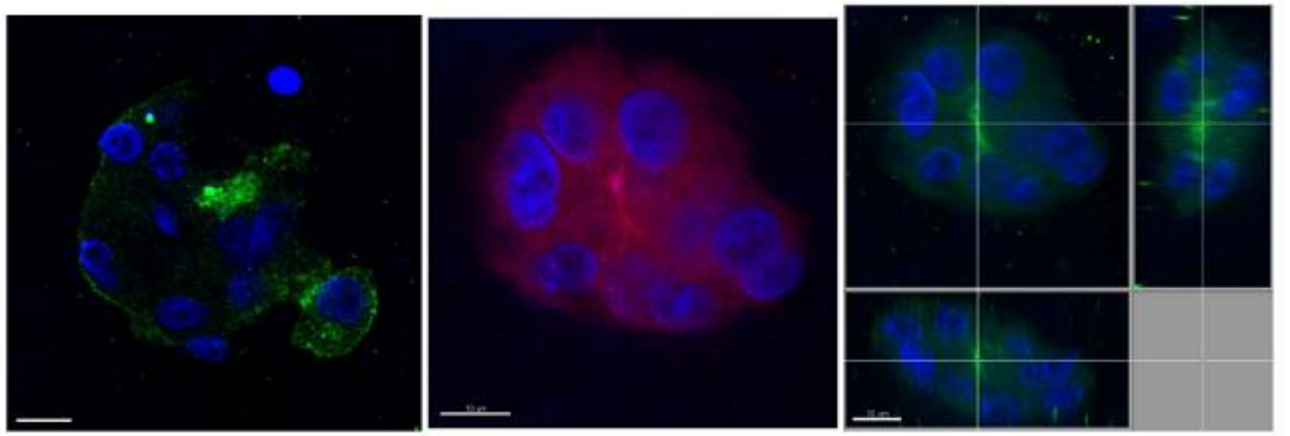
Expressions and activities of metabolic enzymes such as cytochrome P450 have been then studied in comparison with HepaRG-derived hepatocytes known to express high levels of drug metabolism enzymes (Aninat et al., 2006). Expression of CYP3A4 was detected and inducible by rifampicin, although no CYP3A4 activity was yet detected. In ES-derived hepatocytes CYP1A2 expression was also inducible by 3Methylcholanthrene (P1C).

Transfection of HepaRG cells at the progenitor stage with a plasmid encoding human CYP2E1 under a CMV promoter was performed to obtain high CYP2E1 expression levels. We showed that transfection allowed enforced expression of CYP2E1 in both progenitors and differentiated HepaRG cells and that theinsulin-dependent mechanism of CYP2E1 regulation occurs only during the process of hepatocyte differentiation (P1C).This opens new means for drug metabolism and toxicity studies.

ES-derived hepatocytes have been partially validated *in vivo* (1 experiment).
HepaRG were also to engraft and clusters expressing human AA1T and cells expressing CK19 were detected.

The 3D culture conditions used for differentiation of hES cell-derived hepatic progenitors into hepatocytes were optimized. HydroMatrix and nanocellulose promote the expression of ALB and AAT, relevant markers for hepatocyte phenotype.

P6B has demonstrated using HepaRG cells that the 3D culture in nanofibrillar cellulose (NFC) improved the cell polarity and the localization of MRP2 and also MDR1 ABC transporters at the apical membrane of the spheroids, testifying the achieved apical-basolateral cell polarity. The cells cultured in NFC were successfully stained and imaged with confocal microscopy. Nuclei, filamentous actin and some MRP2 and also MDR1 ABC transporters were successfully imaged.



HepaRG cultures in NFC hydrogels. Filamentous actin stained by alexa 594-phalloidin (red), MDR1 (green on the left) and MRP2 (green on the right) immunostaining with alexa 488, and nuclei stained by Hoechst (blue).

The cell cycle progression is controlled by the sequential induction and/or activation of cell cycle regulating proteins including the Cyclin dependent kinase (Cdk) and their regulatory binding partners the cyclins

The search for Cdk11 target genes led to the identification of several genes involved in the regulation of the progression in the G1 phase of the cell cycle. To determine whether the Cdk11-cyclin L complexes could have a role in the entry into and progression through the G1 phase in mature hepatocytes and hepatic progenitors HepaRG cells were used as model.

Protocols to synchronize the progression of progenitor and differentiated HepaRG cells throughout the cell cycle were set up. We demonstrated that while Cdk11 is expressed at a constant level throughout the cell cycle, cyclin L1alpha was barely detectable in quiescent cells but rapidly

induced in early G1 phase with a maximal expression level in mid-G1. These data provide the first evidence for a cell cycle regulation of the cyclin L proteins with an early induction in G1 phase.

Major Conclusions:

- We have generated different tools to characterize, purify expand and differentiate hepatic progenitors and hepatocytes including reporter lentivectors and cell lines and miRNA platforms.
- Novel 3D cell culture methods using matrices, such as nanofibrillar cellulose and peptide nanofibers, have been established. The studies confirmed that the biomaterials maintain the apical and basolateral cell polarity of mature hepatocytes in 3D culture.
- Immortalized lines of mouse and human fetal liver endothelial cells have been established.
- We have developed a chemically defined 2D-platform for the generation of multipotent, endoderm progenitors from several ES cell lines. Under appropriate conditions, these ADE cells can generate liver, pancreas and lung progenitors.
- We have defined conditions for progenitor expansion and cell maturation and to purify hepatic progenitors. Chemically-defined conditions were also defined to generate fetal cholangiocytes from ES-derived progenitors, and from HepaRG progenitors.
- We have defined conditions to obtain more functionally mature hepatocytes.
- Insulin/IRS2 signalling has been identified as a potential target for promoting the proliferation of hepatic precursor cells.

All our data pave the way to generate GMP-hepatocytes for clinical application for the treatment of life-threatening liver diseases. They also will provide conditions to generate expanded hepatocytes either at a fetal stage or at a differentiated stage for toxicology studies.

1.3 POTENTIAL IMPACT AND THE MAIN DISSEMINATION ACTIVITIES AND EXPLOITATION OF RESULTS

Chronic liver disease has a significant impact on the well-being of patients and contributes to the significant morbidity associated with these conditions in the EU. Regenerative medicine comprises the development and application of innovative medical therapies aiming to cure life-threatening and/or chronic diseases or support the regeneration of injured cells, tissues or entire organs. The goal is to avoid organ transplantations, pure technical solutions, or permanent pharmacotherapy. This will therefore lead to a complete paradigm shift in medical treatment, and not merely new technologies and products. As a result of significant progress in biotechnology, regenerative medicine is evolving as a new discipline that could significantly change therapeutic practices.

Medicine, stem cell biology, tissue engineering, nanotechnology, genomic research, material science and system- and process-engineering are coming together to answer fundamental problems of human disease, including trauma and aging and to enable new therapeutic possibilities. The long-term goal is to create new individualized cell-based therapies that will enable autologous regeneration. One area of potential impact for regenerative medicine is the requirement for alternatives to liver transplantation. In particular, novel regenerative cell-based strategies represent some of the most attractive prospects for new therapies of liver failure.

The Liv-ES consortium has established clear objectives and a delivery plan for translating our increased biological understanding of human embryonic stem cells and hepatogenesis into clinical impacts that will benefit both patients, researchers, and perhaps even the economy of the European community. The base of knowledge and methods developed by Liv-ES provide a platform for moving hESC and iPS models towards product development and delivery of cell therapies to the clinic.

Clinical trials of hepatic cells isolated from donor livers have provided convincing evidence that transplantation of these cells can restore liver function, thereby suggesting this approach as a

viable alternative to organ transplantation. However, to implement cell-therapy for chronic liver disease, a renewable source of functional human hepatic cells must be developed. Human embryonic stem cells (hESC) are able to grow indefinitely *in vitro* and differentiate into all cell types of the human body. Although hESC have been differentiated to hepatic cells by various laboratories, the current approaches are not suitable for clinical applications since they rely on animal-derived components which pose risks to human health. The Liv-ES consortium was created to develop reproducible, animal-free conditions for the efficient differentiation of hESC to hepatic progenitors, with the aim of providing the European community with the necessary knowledge and tools to establish a renewable source of transplantable cells for treating chronic liver disease.

Liv-ES has made tremendous progress towards the development of novel reagents and methods for directing the differentiation of hESC to hepatic cells. One of our basic objectives was to create the technological tools to replace animal products in routine culture of hESC. The Liv-ES partners have generated biomaterials which support both the growth and differentiation of hESC and of hepatic progenitor cells. Moreover, we have developed a series of reporter cell lines which are invaluable tools for the identification and purification of cells expressing specific hepatic markers. Other partners have developed a step-wise, chemically-defined protocol for efficiently directing hESC to early-stage progenitors which are committed to the hepatic rather than pancreatic lineage. A renewable source of hepatic cells must expand in vitro: using the reporter tools to identify cells that express specific markers, Liv-ES partners have generated a method to purify hepatic progenitors which can then be expanded in vitro, an important step for future application to cell therapy. The pioneering technology of miRNA has also been employed during the project to identify novel markers of hepatic progenitors. When subjected to further differentiation, these hepatic progenitors yield functional hepatic cells, which secrete albumin and display cytochrome P450 activity, and are capable of engrafting in animal models. Finally, the bipotent HepaRG cell line has been a critical model for LIVES partners to further characterize the culture components and cellular mechanisms which regulate hepatogenesis.

Collectively, the results of Liv-ES provide a means for developing hepatocyte-based therapy as a standardized procedure of regenerative medicine. The findings of Liv-ES have been published widely in journals of high impact; thereby furthering the general knowledge of hESC. Thus, the tools and methodology generated by this project are available to both basic and clinical scientists for translation to human liver diseases. The culture conditions developed and validated by Liv-ES will now enable new therapeutic strategies based on stem cells or engineered tissues for restoring liver function.

In particular our data can be transposed to induced pluripotent stem cell models which opens up not only the approach of cell/gene therapy for the treatment of monogenic disorders or as alternatives to ES cells but also *in vitro* liver disease modeling, as already demonstrated by partners of this project, and the wide market of pharmacotoxicology.

At an economic level, the deliverables provided by Liv-ES provide new opportunities for European biotech companies to develop and market the tools for working in animal-free conditions with pluripotent cells. The methodology developed by Liv-ES will enable EU companies, which have the appropriate facilities to move these procedures to GMP conditions for enabling cell therapy.

1.4 PARTNERS INVOLVED AND COORDINATOR'S CONTACT DETAILS

Part. #	Participant organisation name	Organisation short name	Country	Principal Investigator
1	Institut National de la Santé et de la Recherche Médicale	Inserm	FR	Anne Weber/Georges Uzan Christiane Guillouzo/Anne Corlu
2	The Chancellor, Masters and Scholars of the University of Cambridge	UCAM	UK	Ludovic Vallier
3	The Hadassah Medical Organisation	HMO	IL	Eithan Galun
4	Fundacion de la Comunidad Valenciana Centro de Investigacion Principe Felipe	CIPF	ES	Deborah Burks
5	Vectalys SAS	Vectalys	FR	Pascale Bouillé
6	Helsingin Yliopisto	HU	FI	Arto Urtti Timo Otonkoski
7	Inserm Transfert SA	IT	FR	Christiane Dascher-Nadel

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1.5 PROJECT LOGO AND PUBLIC WEBSITE

www.liv-es.eu

2. USE AND DISSEMINATION OF FOREGROUND

SECTION A (PUBLIC)

Scientific Peer reviewed publications

LIST OF SCIENTIFIC PEER REVIEWED PUBLICATIONS

NO.	Title	Main author	Title of the periodical or the series	Number, date or frequency	Publisher	Place of publication	Year of publication	Relevant pages	Permanent identifiers (if available)	Is/Will open access provided to this publication?
1	Generation of functional hepatocytes from human embryonic stem cells under chemically defined conditions that recapitulate liver development.	Touboul T et al (Inserm A/UCAM)	Hepatology	51(5)	Wiley	online	2010 May	1754-65	http://onlinelibrary.wiley.com/doi/10.1002/hep.23506/abstract;jsessionid=F2A64313E87C3A427F4FCC38A31B9CA7.d03t02	No
2	Cellules souches embryonnaires humaines et iPS - Une source fiable d'hépatocytes fœtaux Robust differentiation of fetal hepatocytes from human embryonic stem cells and iPS	T Touboul et al. (Inserm A/UCAM)	Medecine/sciences	12	-	online	15 December 2010	1061-1066	http://www.medecinesciences.org/index.php?option=com_article&access=doi&doi=10.1051/medsci/201026121061&Itemid=129	Yes
3	Activin/Nodal signalling controls divergent transcriptional networks in human embryonic stem cells and in endoderm progenitors".	Brown S. et al., (UCAM)	Stem Cells	29(8)	Wiley	online	2011 August	1176-85	doi: 10.1002/stem.666.	No
4	Targeted gene correction of α1-antitrypsin deficiency in induced pluripotent stem cells.	Kosuke Y et al., (UCAM)	Nature	478(7369)	Nature	online	2011 October	391-4	doi: 10.1038/nature10424	No
5	Early Cell Fate Decisions of Human Embryonic Stem	Vallier L et al.	PLoS ONE	4(6)	Public Library of Science	online	25 June 2009	e6082	http://www.plosone.org/article/info:doi%2F	Yes

	Cells and Mouse Epiblast Stem Cells Are Controlled by the Same Signalling Pathways	(UCAM/Inserm A)			(PLoS)				10.1371%2Fjournal.pone.0006082	
6	Cyclin-dependent kinase 1 plays a critical role in DNA replication control during rat liver regeneration	Garnier D (Inserm C)	Hepatology	50(6)	Wiley	online	2009 Dec	1946-56	http://onlinelibrary.wiley.com/doi/10.1002/hep.23225/abstract	No
7	Stem cell-derived hepatocytes and their use in toxicology	Guguen-Guillouzo C (Inserm C)	Review in Toxicology	270(1)	Elsevier	online	2010 March	3-9	http://www.sciencedirect.com/science/article/pii/S0300483X09004922	No
8	Highly efficient gene transfer into hepatocyte-like HepaRG cells: New means for drug metabolism and toxicity studies	Laurent V et al. (Inserm C)	Biotechnol. J.	5(3)	Wiley	online	2010 March	314–320	http://onlinelibrary.wiley.com/doi/10.1002/biot.200900255/abstract	No
9	Highly Efficient SiRNA and Gene Transfer into Hepatocyte-like HepaRG Cells and Primary Human Hepatocytes: New Means for Drug Metabolism and Toxicity Studies	Laurent V et al. (Inserm C)	Chapter in Methods in Molecular Biology #107: Cytochrome P450 Protocols “	Third Edition	Humana Press	-	2012	-	-	No
10	Regulation of the G1/S transition in adult liver: Expression and activation of the cyclin dependent kinase Cdk1 in differentiated hepatocytes is controlled by extracellular signals and is crucial for commitment to DNA replication.	Loyer P and Corlu A (Inserm C)	Chapter In “DNA replication Current advances”	-	InTech,	online	2011 August	511-548	http://www.intechopen.com/books/dna-replication-current-advances/regulation-of-the-g1-s-transition-in-adult-liver-expression-and-activation-of-the-cyclin-dependent-k	yes
11	MicroRNA expression patterns and function in endodermal differentiation of human embryonic stem cells	Tzur G et al. (HMO)	PLoS One	3(11)	Public Library of Science (PLoS)	online	18 Nov 2008	e3726	http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0003726	Yes
12	Increased microRNA activity in human cancers	Israel A et al. (HMO)	PLoS One	4(6)	Public Library of Science (PLoS)	online	25 June 2009	e6045	http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0006045	Yes
13	Comprehensive gene and microRNA expression profiling reveals a role for microRNAs in human liver	Tzur G et al. (HMO)	PLoS One	4(10)	Public Library of Science (PLoS)	online	20 Oct 2009	e7511	http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0007511	Yes

	development									
14	The oncofetal H19 RNA connection: Hypoxia, p53 and cancer	Matouk IJ et al. (HMO)	Biochim Biophys Acta	1803(4)	Elsevier	online	1 Feb 2010	443-451	http://www.sciencedirect.com/science/article/pii/S016748891000011X	No
15	Accelerated carcinogenesis following liver regeneration is associated with chronic inflammation-induced double-strand DNA breaks	Barash H et al. (HMO)	Proc Natl Acad Sci U S A	107(5)	National academy of sciences	online	25 Jan 2010	2207-12	http://www.pnas.org/content/early/2010/01/14/0908867107	No
16	An important role for CDK2 in G1 to S checkpoint activation and DNA damage response in human embryonic stem cells	Neganova I et al (CIPF)	Stem Cells	29 (4)	Wiley	online	Apr 29 2010	651-69	http://onlinelibrary.wiley.com/doi/10.1002/stem.620/	Yes
17	Inhibition of PTP1B restores IRS1-mediated hepatic insulin signaling in IRS2-deficient mice	González-Rodríguez A et al	Diabetes	59(3)	American Diabetes Association	online	March 2010	588-99	http://diabetes.diabetesjournals.org/content/59/3/588.long	Yes
18	Development of a Human Extracellular Matrix for Applications Related with Stem Cells and Tissue Engineering	Escobedo-Lucea C et al (CIPF)	Stem Cell Reviews and Reports	8 (1)	Springer-Verlag	online	Mar 8 2012	170-83	http://www.springerlink.com/content/jp6m01x657455708/?MUD=MP	Yes

Manuscripts in preparation

MANUSCRIPTS IN PREPARATION

NO.	Title	Main author	Title of the periodical or the series targeted	Number, date or frequency	Publisher	Place of publication	Publication planned for	Relevant pages	Permanent identifiers (if available)	Is/Will open access provided to this publication?
1	Purification and differentiation of integration-free human ES cell-derived hepatic progenitors using Integrase Deficient Lentivectors	Yang G Inserm A/UCAM/ Vectalys/ CIPF	Submitted	-	-	-	-	-	-	No

2	Involvement of TGFb pathway in the reversion program of hepatocyte-HepaRG cells into bipotent progenitors"	Dubois-Pot H et al (inserm C)	Hepatology		Wiley		2012			No
3	Inhibition of Activin/Nodal signalling is necessary for pancreatic differentiation of human pluripotent stem cells.	Candy Cho / Nick Hannan (UCAM)	Diabetologia	-	EASD	-	-	-	-	No
4	Derivation of hepatocyte-like cells from human pluripotent stem cells.	Nick Hannan (UCAM)	Nature Protocol	-	Nature	-	-	-	-	No
5	Wnt-dependent propagation and specification of human definitive endoderm cells	Lundin K., Toivonen S. (HU)	NYD	-	-	-	-	-	-	-
6	Native plant cellulose nanofiber hydrogels as a scaffold for 3D cell spheroid formation in culture	M Bhattacharya (HU)	J Control Rel	-	Elsevier	-	2012	-	-	No
7	Peptide nanofiber hydrogel induces formation of bile canalicular structures in 3D cultures of hepatic cell line	M. Malinen (HU)	Tissue Engineering	-	Mary Ann Liebert	-	2012	-	-	No
8	Regulation of mir 122 expression	Giladi H (HMO)	Hepatology	-	Wiley	-	2012	-	-	No
9	MicroRNA editing	Mandelbaum (HMO)	Nature Immunology	-	Nature	-	2012	-	-	No
10	IRS2-mediate insulin signalling is required for human hepatocyte differentiation in vitro	Noon LA et al (CIPF)	Hepatology	-	Wiley	-	2012	-	-	No

Dissemination Activities (conferences etc.)

No.	Type of activities	Main leader	Title of the activity (e.g. conference title)	Title of the presentation	Date	Place	Type of audience	Size of audience	Countries addressed
1	Website	IT	Project website	www.liv-es.eu		-	General public, scientific community, Industry	-	International
2	Symposium	IT	Liv-ES Symposium	Embryonic Stem Cells: New Tools for Treating Human Liver Disease	12 - 13 March 2012	Paris, France	Scientific community, Industry, Medical community	96	Europe
3	Press Release	IT	Project launch	Launch of the Liv-ES project	February 2009	-	Scientific community, Industry, Medical community	-	Europe/International
4	Conference poster	Inserm A	SFTCG Société francophone de Thérapie cellulaire et génique	Differentiation of human pluripotent stem cells into hepatic progenitors	June 17 2009	Giens, France	Scientific Community	70	France
5	Conference - Poster	Inserm A	ISSCR 7th Annual Meeting	Generation of human embryonic stem cells-derived hepatocytes: in vitro and in vivo functional studies	July 8-11, 2009	Barcelona, Spain	Scientific Community	5000	International
6	Conference oral	Inserm A	AFEF French Association for the study of liver	Caractérisation *in vivo* et purification de progéniteurs hépatiques dérivés de cellules souches embryonnaires humaines	Sept 30-oct2 2009	Paris France	Scientific Community	60	France

7	Conference oral	Inserm A	AASLD American Association for the study of liver diseases	Generation of hepatocytes from human embryonic stem cells: in vitro &in vivo functional studies	Nov 3 2009	Boston USA	Scientific Community	4000	International
8	Invited conference	Inserm A	AFEF French Association for the study of liver	Nouvelles approches de therapie cellulaire dans le foie	Sept 2010	Marseille France	Scientific Community	80	France
9	Conference oral	Inserm A	AASLD American Association for the study of liver diseases	Purification&differentiation of hepatic progenitors generated from human ES cells	Nov 1, 2010	Boston USA	Scientific Community	4000	International
10	Invited conference	Inserm A	EASL European Association for the study of Liver	Human hepatic progenitors: from ES cells to foetal cells	April 18 2010	Vienne Austria	Scientific Community	2000	Europe
11	Conference -poster	Inserm A	ESCGT European Society for Cell and Gene Therapy	Lentiviral vector mediated purification of hepatic progenitors differentiated from human embryonic stem cells	Oct 22-25 2010	Milano Italy	Scientific Community	1000	Europe
12	Conference – Oral Presentation	Inserm A	e-chips European conference on human iPS stem cell reprogramming	Pluripotent stem cells and hepatic differentiation	Avril 18, 2011	Paris France	Scientific Community	100	Europe
13	Conference – Oral invited	Inserm A	HEPARG workshop Biopredic International	Human Pluripotent Stem cells:models for hepatocyte production	Sept 23, 2011	Rennes France	Scientific Community	150	International
14	Conference poster	Inserm A	ISSCR	Hepatic progenitors can be purified upon differentiation of human embryonic stem cells.	June 15-18, 2011	Toronto Canada	Scientific Community	5000	International
15	Conference poster	Inserm A	ESCGT	Lentiviral vector	Oct 27-31	Brighton	Scientific	1000	International

				mediated purificationof hepatic progenitors differentiated from human ES cells	2011	England	Community		
16	Conference poster	Inserm A	Stem Cell Programming and Reprogramming	Fluorescent activated cell sorting-mediated purification of human embryonic stem cell-derived hepatic progenitors.	Dec. 8 - 10, 2011	Lisbon, Portugal	Scientific Community	250	International
17	Conference oral presentation	Inserm A	Workshop: Current Trends in Biomedicine. Liver and Pancreas: From Development to Disease	Purification of human embryonic stem cell-derived hepatic progenitors.	November 14-16, 2011	Baeza, Spain	Scientific Community	250	Europe
18	Conference oral presentation	Inserm A	Liver Down Under Conference	Purification of human embryonic stem cell-derived hepatic progenitors.	November 28-December 02, 2011.	Perth Australia	Scientific Community	1500	International
19	Symposium – Oral presentation	Inserm A	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Purification of ES-derived hepatic progenitors	March 12-13, 2012	Paris, France	Scientific	100	Europe
20	Symposium – Poster	Inserm A	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Differentiation of human ES cell-derived hepatic progenitors into hepatocytes	March 12-13, 2012	Paris, France	Scientific	100	Europe
21	Symposium – Poster	Inserm B	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Purification and characterization of fetal liver sinoisidal endothelial cells	March 12-13, 2012	Paris, France	Scientific	100	Europe
22	Conference - Poster	Inserm C	3ème congrès Génomique Fonctionnelle du Foie	Transfert de gènes dans les cellules d'hépatome humain HepaRG	March 11-12 2010	Rennes, France	Scientific	200	France
23	Conference – Poster presentation	Inserm C	New Developments in Cell-Based In-Vitro	Preferential hepatocyte	18-20th May 2011.	Saarbrucken, Germany	Scientific	90	Europe

			Testing & 3rd Annual Quasi-Vivo User Group Meeting	differentiation directed by shape constraint in stem-like hepatic HepaRG cells					
24	Conference – oral presentation	Inserm C	New Developments in Cell-Based In-Vitro Testing & 3rd Annual Quasi-Vivo User Group Meeting	Tissue culture and regenerative medicine	18-20th May 2011.	Saarbrucken, Germany	Scientific	90	Europe
25	Conference- oral invited	Inserm C	HepaRG workshop Biopredic International	Reprogramming and differentiation of HepaRG cells	Sept 23, 2011	Rennes France	Scientific	150	International
26	Conference- Poster	Inserm C	HepRG workshop Biopredic International	Preferential hepatocyte differentiation directed by shape constraint in stem-like hepatic HepaRG cells	Sept 23, 2011	Rennes France	Scientific	150	International
27	Conference Oral presentation Invited speaker	Inserm C	Gen2Bio- Rencontres de biotechnologie	Revues des technolmogies d'électroporation	March 29 2012	Lorient, France	Scientific	50	France
30	Conference-Poster	Inserm C	4ème colloque «Génomique fonctionnelle du Foie»	Reprogrammation des hépatocytes HepaRG en cellules souches/ progénitrices	14-16 mars 2012	Bordeaux, France	Scientific	100	France
31	Conference -poster	Inserm C	ISSCR 10th annual meeting,	reprogramming of transformed HepaRG hepatocytes into bipotent progenitors	June 13-16, 2012	Yokohama, Japon	scientific	5000	International
32	Symposium – Oral presentation	Inserm C	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Functional requirements for metabolic studies in hepatocytes	March 12-13, 2012	Paris, France	Scientific	100	Europe
33	Symposium – Poster	Inserm C	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Mechanisms of HepaRG hepatocytes reversion into bipotent progenitors	March 12-13, 2012	Paris, France	Scientific	100	Europe
34	Conference - Poster	UCAM	ISSCR 7th Annual	Identification of	July 8-11, 2009	Barcelona,	Scientific	5000	International

			Meeting	Transcription Factor Networks Involved in Hepatic Specification of hESCs		Spain	Community		
35	Conference - Poster	UCAM	ISSCR 7th Annual Meeting	Production of multipotent Anterior Definitive Endoderm from human pluripotent stem cells	July 8-11, 2009	Barcelona, Spain	Scientific Community	5000	International
36	Conference - Oral presentation	UCAM	Stem cells in development and diseases	Production of Multipotent Foregut Endoderm from Human Pluripotent Stem Cells	September 11-14 2011	Berlin Germany	Scientific Community	200	International
37	Symposium – Oral presentation	UCAM	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Signalling pathways controlling patterning of human endoderm in vitro	March 12-13, 2012	Paris, France	Scientific	100	Europe
38	Symposium – Oral presentation	HMO	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	MicroRNAs in development: Focus on the liver	March 12-13, 2012	Paris, France	Scientific	100	Europe
39	Conference – Oral Presentation	HMO	Falk Symposia	Accelerated carcinogenesis following liver regeneration	26 – 27 Jan 2012	Hamburg, Germany	Scientific Community	300	Europe
40	Course	HMO	Gene therapy Series	Lentivectors and others	June 2011	Germany, Germany	Students (PhD MSc)	100	Europe
41	Conference – Oral Presentation	HMO	ECCO Annual Meeting	Liver inflammation	26 June 2010	Oslo, Sweden	Scientific Community	2000	International
42	Conference – Oral Presentation	HMO	Inflammation & Cancer meeting	Liver inflammation and cancer	24 March 2012	Jerusalem, Israel	Scientific Community	250	International
43	Conference – Oral Presentation	CIPF	Interbio	The Role of Insulin Signalling in the Differentiation of	18-21 January, 2010	Valencia, Spain	Scientific Community	60	Europe

				hESC to Hepatocytes					
44	Conference – Oral Presentation	CIPF	CIBERDEM annual meeting	The Role of insulin signalling in hepatocyte differentiation.	Oct 21-23, 2011	Barcelona, Spain	Scientific Community	120	Europe
45	Conference – Poster Presentation	CIPF	Liver and Pancreas: From Development to Disease	IRS2-mediate insulin signalling is required for human hepatocyte differentiation of HepaRG cells	Nov 14-16 2012	Baeza Spain	Scientific Community	80	International
46	Conference – Oral Presentation	CIPF	Liv-ES Symposium Embryonic Stem Cells: New Tools for Treating Human Liver Disease	Insulin and IRS2 are required for human hepatocyte differentiation in vitro	March 12, 13 2012	Paris, France	Scientific Community	120	International
47	Conference – Oral Presentation	Vectalys	SBS 16th Annual Conference Advancing the science of drug discovery	Lentiviral vectors as a tool for cell engineering in drug discovery	April 11-15, 2010	Phoenix, Arizona, USA	Scientific Community, Biotech Industry	2 000	International
48	Symposium –Oral Presentation	Vectalys	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Lentiviral vectors, a tool for identification and selection of human stem cell derived cells	March 12-13, 2012	Paris, France	Scientific	100	Europe
49	Conference – Poster	HU - A	ISSCR 7th Annual Meeting	Optimisation of culture conditions for differentiation of human pluripotent stem cells to hepatic and pancreatic progenitors.	July 8-11, 2009,	Barcelona, Spain	Scientific Community	5000	International
50	Conference – Poster	HU - A	ISSCR 8th Annual Conference	Maintenance of human pluripotent stem cell derived definitive endodermal progenitor cells in long-term culture depends on	June 16-19, 2010,	San Francisco, USA	Scientific Community	5000	International

				persisting pluripotent cells					
51	Workshop - Poster	HU - A	EMBO Workshop on Beta cell differentiation and regeneration	Differentiation of human pluripotent stem cells into pancreas precursors – focus on the definitive endoderm induction	Feb26 – March 1, 2009,	Peebles, UK	Scientific Community	200	Europe
52	Workshop - Oral	HU - A	EMBO Workshop on Beta cell differentiation and regeneration	Maintenance of pluripotent stem cell derived definitive endodermal progenitor cells in long-term culture	Feb26 – March 1, 2009,	Peebles, UK	Scientific Community	200	Europe
53	Conference – Poster	HU - A	ISSCR 9th Annual Meeting	Development of 3D methods for the differentiation of hepatocyte like cells from human pluripotent stem cells	July 8-11, 2011,	Toronto, Canada	Scientific Community	5000	International
54	Symposium - Poster	HU-A	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Direct differentiation of patient-specific iPSC to model liver and pancreas diseases	March 12-13, 2012	Paris, France	Scientific	100	Europe
55	Symposium - Poster	HU-A	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Wnt-dependent propagation and specification of human definitive endoderm cells	March 12-13, 2012	Paris, France	Scientific	100	Europe
56	Symposium - Poster	HU-A	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Differentiation of hepatocyte-like cells from human pluripotent stem ells in vitro using a definitive lectin-based VPU matrix	March 12-13, 2012	Paris, France	Scientific	100	Europe
57	Conference - Oral	HU-B	MMC14	3D culture of hepatic cells in biomaterials	August 18, 2011	Helsinki, Finland	Scientific Community	300	Europe

58	Conference - Poster	HU-B	PSWC/AAPS Annual Meeting	Generation of functional three-dimensional liver model for drug development	November 14-18, 2010	New Orleans, USA	Scientific Community	-	International
59	Conference - Oral	HU-B	Helsinki Drug Research Conference	3D cell cultures in drug research	September 20, 2011	Helsinki. Finland	Scientific Community	170	International
60	Seminar - Oral	HU-B	Johns Hopkins University, Baltimore	Hepatic 3D cell cultures in biomaterials	August 4, 2011	Baltimore, MD, USA	Scientific Community	30	USA
61	Symposium – Oral presentation	HU-B	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	3D cultures of human stem cell derived cells	March 12-13, 2012	Paris, France	Scientific	100	Europe
61	Symposium – Poster	HU-B	Liv-ES Symposium - Embryonic stem cells: New tools for treating human liver diseases	Generation of functional three-dimensional liver cell culture model for drug development	March 12-13, 2012	Paris, France	Scientific	100	Europe
62	Conference - Poster	HU-B	7th FinBioNet PhD Student Symposium Five Senses and Science	Generation of Three-dimensional Hepatic Cell Cultures for Drug Discovery	2011	Helsinki, Finland	Scientific Community	100	Finland
63	Conference - Poster	HU-B	XX Helsinki Drug Research Conference	Generation of Three-dimensional Hepatic Cell Cultures for Drug Development.	September 20, 2011	Helsinki, Finland	Scientific Community	170	International
64	Conference - Poster	HU-B	PSWC 2010 Congress for Students and Postdoctoral Fellows	Generation of Functional Three-dimensional Liver Cell Culture Model for Drug Development.	November 2010	New Orleans, LA, USA	Scientific Community	100	International
65	Conference - Poster	HU-B	The meeting of Globalization of Pharmaceutics Education Network (GPEN)	Generation of Functional Three-dimensional Liver Cell Culture Model for Drug Development.	November 2010	Chapel Hill, NC, USA	Scientific Community	200	International

66	Conference - Poster	HU-B	PharmSciFair	Three-dimensional liver cell culture as a model for hepato-biliary transport studies	2009	Nice, France	Scientific Community	300	International

SECTION B (CONFIDENTIAL OR PUBLIC)

Applications for patents, trademarks etc.

TEMPLATE B1: LIST OF APPLICATIONS FOR PATENTS, TRADEMARKS, REGISTERED DESIGNS, ETC.					
Type of IP Rights:	Confidential Click on YES/NO	Foreseen embargo date dd/mm/yyyy	Application reference(s) (e.g. EP123456)	Subject or title of application	Applicant (s) (as on the application)
Patent	No	-	EP20090306136	Method for hepatic differentiation of definitive endoderm cells	INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM) (for all designated states except US) CAMBRIDGE ENTERPRISE LIMITED (for all designated states except US) TOUBOUL, Thomas (for US only) VALLIER, Ludovic (for US only) WEBER-BENAROUS, Anne (for US only)

Exploitable Foreground generated during the project

Type of Exploitable Foreground	**Description of exploitable foreground**	Confidential Click on YES/NO	Foreseen embargo date dd/mm/yyyy	**Exploitable product(s) or measure(s)**	**Sector(s) of application**	**Timetable, commercial or any other use**	**Patents or other IPR exploitation (licences)**	**Owner & Other Beneficiary(s) involved**
Commercial exploitation of R&D results	Tissue specific ready to use lentiviral vectors	YES	-	Scientific community and pharma companies and CRO companies	Target Validation	2013	No protection	Vectalys