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Innovative strategies to generate human hepatocytes for treatment of metabolic Liver diseases: Tools for personalized cell therapy

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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. Hepatocyte transplantation has provided encouraging results and thus, suggests that such cell-based therapies may represent viable alternatives to OLT. However, in addition to the complication imposed by the shortage of organ donors, there is a lack of standardized procedures to assess the quality of primary hepatocyte preparations and *in vitro* these cells cannot be expanded. Autologous transplantation of genetically corrected cells represents an alternative to allotransplantation for metabolic diseases as it circumvents the problems of immunosuppression when long-term hepatocyte engraftment is necessary. However, this approach requires a lobectomy representing 20% of the liver to isolate hepatocytes, which is not devoid of risks in patients with diseases such as Familial Hypercholesterolemia. Therefore to implement cell-therapy for chronic liver diseases, a renewable source of functional human hepatic cells must be developed.

Thus, development of safe, cost-effective methods for treating chronic liver disease reflects a health priority. Although hESC have been differentiated to hepatic cells by various laboratories, the production of safe and fully functional, human hepatocytes from hESC under GMP conditions for clinical applications remains to be achieved. Reprogramming of human somatic cells to a pluripotent state offer the advantage that they these induced pluripotent stem cells can be produced via non-invasive procedures in patients. Therefore, hiPSC represent an ideal source for generating patient-specific and disease-specific cells for clinical applications.

The consortium *InnovaLiv* has pursued the project **"Innovative strategies to generate human hepatocytes for treatment of metabolic liver diseases: tools for personalized cell therapy"** to launch European hES cell-based therapy for patients with life threatening metabolic diseases. Innovaliv has developed scale-up conditions for Spanish undifferentiated VAL9 cells and other pluripotent stem cells and a master differentiation protocol, both based on animal-free, cGMP-grade reagents. In addition other GMP-compliant/compatible protocols have been defined to differentiate VAL9 cells into functional hepatocytes. These conditions allow also differentiation of other pluripotent stem cells. Cells derived from two of these protocols have been validated in vivo for safety issues and in vivo for cell functions and are able to proliferate after engraftment and to rescue mice with acute failure with no evidence of tumorigenicity.

GMP-compliant conditions for cryopreservation of VAL9-hepatoblasts and hepatocytes have been defined, as well as for other pluripotent stem cell lines. Finally, conditions for large-scale production of hESCs in suspension have been defined.

Some partners have generated several iPSC lines either with integrative or non-integrative methods from several patients with metabolic diseases: Crigler-Najjar (CN), Familial Hypercholesterolemia (FH) and Hemophilia B (HB). They have expanded, cryopreserved and differentiated them into hepatocytes. They have also employed a pioneering technology of targeted recombination to correct a mutated gene. These corrected clones expressed the protein and displayed a phenotypic activity. Other new technologies for gene correction by homologous recombination were also developed. Different rat models have been generated to evaluate iPSC-hepatocytes engraftment, function and safety. Normal iPSC-hepatocytes and corrected CN-iPSCs were transplanted and BOTH resulted in a significant long-term correction of hyperbilirubinemia with no evidence of tumorigenicity (test period=6 months). Finally, a preclinical dossier was prepared for safety and quality control standards for the cells since compliance to regulations is critical for the application of our protocol to clinical trials. Criteria for selecting patients were defined.

Collectively, the results of *InnovaLiv* provide robust conditions for developing hepatocyte-based therapy as a standardized procedure of regenerative medicine. Thus, the tools and methodology generated by this project are available to both basic and clinical scientists for translation to human liver diseases. Liver is also the major organ for eliminating xenobiotics and therefore, plays a key role in pharmacokinetics and toxicology. Therefore iPSCs generated from patients with metabolic disease, in particular FH, since LDL receptor, the gene responsible for the disease, is involved in virus entry will serve as drug screening model.

SUMMARY DESCRIPTION OF PROJECT CONTEXT AND OBJECTIVES

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, there is a lack of standardized procedures to assess the quality of primary hepatocyte preparations and these cells 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.

Autologous transplantation of genetically corrected cells represents an alternative to allotransplantation for metabolic diseases as it circumvents the problems of immune rejection and immunosuppression when long-term hepatocyte engraftment is necessary. However, this approach was evaluated once 20 years ago and was not repeated because (i) it requires a lobectomy representing 20% of the liver to isolate hepatocytes, which is not devoid of risks in patients with diseases such as Familial Hypercholesterolemia, (ii) autologous hepatocytes can be transplanted only once, (iii) the efficacy of genetic correction depends on the efficacy of hepatocyte transduction at the time of their isolation.

Stem-cell based regenerative medicine has emerged as a viable approach for the treatment of chronic liver diseases such as Hypercholesterolemia and Hemophilia B. Given the shortage of donor livers in the EU countries, there is a large demand for transplantable hepatocytes. Although hESC and hiPSC represent potential sources of cell-based therapies, the translation of these to human diseases requires the scale-up production and banking of large numbers of hepatocytes under conditions compatible with clinical trials. In the last few years, several groups have reported the differentiation of hES cells into hepatic cells using diverse culture systems. However, most of 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. Thus the lack of large-scale, GMP-grade methods for the culture of hESC/iPSC presents a serious challenge to the progression of new therapies to clinical testing. In addition, the lack of well-characterized cryopreservation protocols has limited the reproducibility of results between laboratories. Moreover, the proliferative capacity of hESC-derived hepatocytes must be carefully controlled for clinical applications since some reports have shown that, once transplanted, they can produce teratomas or adenocarcinomas *in vivo*.

Advances in cellular reprogramming have demonstrated that human somatic cells can be converted to a pluripotent state. These induced pluripotent cells (hiPSC) closely resemble hESC in that they can be expanded in culture and can be differentiated to a variety of cell types and offer the additional advantage that they can be produced via a skin biopsy or blood cells, a non-invasive procedure in patients. Therefore, hiPSC also represent an ideal source for generating patient-specific and disease-specific cells not only for drug screening but also for clinical applications after in vitro correction of the mutated gene (personalized medicine). However there are some specific safety issues related to these cells and, for example, more rigorous methods are needed to monitor

genetic and epigenetic changes that may occur during the long-term culture of pluripotent cells and their differentiation products.

The overall objective of ***InnovaLiv*** has been to address the technical challenges of stem-cell based therapies by developing innovative methods and tools for GMP large-scale maintenance and expansion of undifferentiated pluripotent stem cells and for their differentiation to functional human hepatic cells.

A further objective has been to generate hiPSC lines from patients with inherited metabolic liver diseases (Familial Hypercholesterolemia, Hemophilia B and Crigler-Najjar) to develop innovative tools for correcting *in vitro* the genetic defects, which underlie the hepatic dysfunction. The corrected hiPSC have then be differentiated into hepatocytes, thereby creating in vitro models of liver diseases for basic research and drug screening. These novel strategies based on hiPSC represent a unique opportunity to develop a cell-based therapy, which circumvents the need for immuno-suppression in patients.

WP1 Development of scale-up conditions for production of GMP-grade human hepatocytes
The global objective of WP1 has been to define an optimal protocol for large-scale differentiation of hESC to hepatocytes in a GMP facility. The use of European hESC lines has been a priority for WP1. Safety and functional tests have been conducted at different phases of this development process. To obtain scale-up conditions for production of GMP-grade human PSC-derived hepatocytes several specific objectives have been pursued:

- Identification of a scalable culturing system of GMP compliant for undifferentiated hESC that maintain the pluripotency of hESC and allow efficient differentiation to hepatocytes.
- Identification of a protocol for differentiating hESC (that are cultured in a scalable GMP compliant culturing system) to hepatocytes of high purity and with hepatic features.
- Develop cryopreservation technology for hESC-derived hepatocytes
- Develop scale-up conditions for cryopreservation of hESC-derived hepatocytes
- Substitute all reagents in the differentiation- and cryopreservation protocols to GMP compliant reagents.
- Assessment of the tumorigenesis risk of hESC-derived hepatocytes
- Develop industrialised scale-up conditions of hESC-derived hepatocytes

WP2 – Production of tools for hiPSC-based therapy of genetic liver diseases
Human somatic cells such as fibroblasts can be reprogrammed into induced pluripotent stem cells (hiPSC). hiPSC offer the advantage of a patient-specific gene/cell approach that avoids cell rejection and the need for immunosuppression. hiPSC provide an excellent model to establish proof-of-concept that genetic defects can be corrected in vitro by vectors that express the missing or defective genes. WP2 will focus on the inherited metabolic liver diseases of Familial Hypercholesterolemia (FH), Hemophilia B, and Crigler-Najjar. Human iPSC from patients with FH, Hemophilia B and Crigler-Najjar will be generated and then differentiated to hepatocytes using the conditions and reagents developed by WP1. The resulting diseased hepatocytes will be compared to hESC-derived hepatocytes in terms of genome integrity, metabolic activity and in vivo safety. Correction of genetic defects will be achieved by the SIN lentiviral vectors encoding the human correcting proteins or by Zinc Finger Nuclease approach, which allows the insertion of the correction cassette to be targeted to a "safe harbor" genome locus. Restoration of hepatocyte functions will be assessed. The ultimate objective will be the scale-up production of these hiPSC lines as human disease models.

WP3 – Assessment of engraftment capacity and functionality of hiPSC
The development of patient-specific hiPSC in WP2 will allow proof-of-concept that hiPSC-derived hepatocytes bearing a genetic rescue-of-function can restore hepatic function *in vivo*. FH-hiPSC-derived hepatocytes bearing a genetic correction will be transplanted into livers of Rag2-/-□c-/-uPA-immunodeficient mice. In this model, transplanted hepatocytes repopulate the host tissue, thereby regenerating hepatic function in vivo. HB-hiPSC-derived hepatocytes will be transplanted into the liver of FIX KO newborn mice to quantify FIX production. CN-hiPSC-derived hepatocytes will be

transplanted after correction into the liver of the newborn Gunn rats and bilirubin levels will be assayed in serum of transplanted animals.

WP4 – Clinical use and Compliance with regulatory and ethical legislation
WP4 tasks ensure that regulatory and ethical issues related to the proposed clinical trial are addressed adequately. Cell products derived from WP1 should meet the safety, manufacturing procedures, and quality issues according with the requirements of regulatory and ethical legislation. In general, to obtain the authorization of the Spanish Regulatory Agency (AEMPS) for performing a clinical trial, the quality of the drug, its safety and its expected clinical efficacy has to be properly documented. *InnovaLiv* will make use of manipulated hESC (VAL9), differentiated into hepatocytes. The manipulations done on these cells before their clinical use are beyond the borders accepted in a conventional cell transplantation procedure. Consequently Innovaliv Cells are considered a pharmaceutical for an advanced cell therapy. A tentative pre-clinical dossier should be defined that includes the key issues and SOPs related to the manufacturing, quality, safety and efficacy of the VAL9 and VAL9-derived hepatocytes preparation. In the manufacturing process the product must demonstrate batch to batch consistency, cell purity, traceability and reproducibility throughout all the stages of cell production (hES expansion and differentiation). To ensure that cells can be produced in a reproducible manner (GMP) and the reproducibility of any additional procedure the cells might be involved (i.e., freezing-thawing / transport). A GMP manufacturing license by Cellectis (D4.3) should be delivered. In terms of efficacy results show the hepatic metabolic functionality of the cells in vitro. The preliminary demonstration of the VAL9- hepatocytes in vivo efficacy was assessed by transplanting, these cells, generated by another GMP-compatible protocol, into a mouse model of acute liver failure helping to recover the hepatic functionality of the host liver. Finally, the follow up in Spain of a list of selected paediatric patients with inherited hepatic metabolic diseases has been performed. One of these candidates should be included in the clinical trial expecting that cell therapy will result in clinical improvement and better quality of life for the patient.

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.
For translation to clinical procedures, culturing systems including media, cytokines and matrixes must be chemically defined to avoid the use of animal products, feeder cells, and chromatin modifiers.
Moreover, the proliferative capacity of hESC-derived cells 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.

Thus, some of the principal objectives of the *InnovaLiv* consortium have focused on the development of reagents and methods to standardize the culture and differentiation of hESC to hepatic cells in GMP conditions.

Workpackage 1 of the *InnovaLiv* experimental plan has focused on Scale-up production of clinical-grade hepatocytes from a European hESC-line

1. Deviation plan

The original work plan proposed to focus the *InnovaLiv* studies on candidate European hESC lines derived in Sweden or Spain, the advantages and disadvantages of which were discussed at the kick-off meeting and during a meeting in Cambridge in January 2012 with the aim to 1) choose a hESC line, 2) discuss a culturing system for maintaining hESC that is suitable for up-scaling and 3) set up a detailed strategy for characterisation work that should be performed by the different partners investigating hepatocyte differentiation protocols. At this time point it was clarified that Cellectis was not approved to work with VAL9 from the Spanish stem cell bank due to the status of a commercial company. Therefore it was decided that the Swedish SA181 would be used for this project. However, end of March 2012, it became evident that SA181 was anonymised, and that this prevents this cell line to be used in clinical activities. Given the legal complications with SA181, the hESC line VAL9 derived in Institute of Partner 3 was finally selected as the prototype hESC line to be scaled-up and differentiated to hepatic lineage under GMP conditions. This cell line was deposited in 2009 with the national bank and additionally forms part of the European Human Embryonic Stem Cell Registry.

To obtain the VAL9 line, individual partners of *InnovaLiv* were required to submit specific applications to the Spanish national stem cell bank including an authorization from institutional ethics committee. However, this process has taken longer than anticipated due to the change of personnel in Spain's health ministry. Moreover further discussions regarding Cellectis having access to VAL9 delayed of a few months the formal signing of the MTA. Finally, beginning of December 2012, the MTA for VAL9 cells was sent to the Spanish stem cell bank by Cellectis and vials of VAL9 cells were subsequently sent to Cellectis. In summary, due to legal issues and authority complications the GMP-hESC-line VAL9 was not available for all partners until one year into the project. This has delayed the first deliverables and mile stones; "Master protocol for differentiation of hESC to hepatocytes for transition to GMP compliance" (D1.2), "Choice of differentiation protocol for evaluation" (MS1) and "Evaluation of differentiation efficacy" (MS2). This delay has, in turn, slowed the tasks of WP4, which is dedicated to securing the authorizations and agreements necessary for the transplantation of hESC-derived hepatocytes in a patient with end-stage, metabolic liver disease.

During the first 12 months of the project, the emphasis has been on working on hESC-line available in each lab and differentiating it to hepatocytes using different protocols.

The coordinated effort to define a GMP-compatible medium and matrix for the maintenance, amplification, and cryopreservation of VAL9 cells has taken more time than outlined in the original workplan. Indeed, according to the German Stem Cell Act CellGenix (P6) is not allowed to import and use human embryonic stem cells derived after May 1st 2007. Therefore, P6 is not permitted to use the VAL9 cell line as this cell line was derived after the effective date. Instead, CellGenix received the authorization to import and use Swedish cell lines that had been established between 2002 and 2005. They chose SA181 for which Cellectis results indicated that hepatic differentiation was most robust as well as an iPSC line for their training and to test their different products.

2. Culture conditions testing and characterization of VAL9 cells

Protocols for thawing and culturing VAL9 has been transferred by P3 to the different partners who have been provided with control material for quantitative PCR comparison of their VAL9 cultures to VAL9 maintained on feeders.

When VAL9 cells were received VAL9 cells were first grown on non-GMP compatible matrixes MEF and Geltrex then successfully adapted to gelatin in the different labs concerned (P1A, P2).

Pluripotency was analysed at different passages by different methods quantitative PCR (Oct4, and Nanog), immuno-cytochemistry (Oct4, SSEA4, and Tra-1-60) and FACS (SSEA4, and Tra-1-60) in the different labs involved. Karyotype was a diploid normal XX one.

Teratoma formation was observed in immunodeficient mice injected with VAL9 cells confirming the pluripotency in vivo of the cells.

Different media and conditions to expand and bank VAL9 cells were first tested by SMEs partners (P5 and P6). Cellectis feeder-free culturing system, DEF-CS has shown to be a robust culturing system to expand VAL9 for more than 29 passages with stable karyotype and retained pluripotency. In collaboration with Novo Nordisk, Cellectis has been developing a GMP version of the DEF-CS which has become fully defined and cGMP in 2014 and denoted DEF-XF. Another option has been to test the commercial available cGMP E8-media provided by Life Technology.
Partner 2 (UCAM) had experience culturing and expanding VAL9 hESC using the E8 media and gelatine / FBS as coating. DEF-XF and the E8 culturing system were compared in different coating conditions on VAL9 hESC but in P5 laboratory, the E8 system was not able to maintain VAL9 hESC cultures for more than three passages and cells could not be efficiently differentiated to hepatocytes. Consequently, Cellectis had to change the culture system from E8 to DEF-XF, which had become available as GMP for the Innovaliv project to use in the frame of Cellectis collaboration with NovoNordisk and which also reproducibly promoted differentiation of VAL9 cells into hepatocytes. Thus, the development of a new prototype, clinical-grade medium by P5 has been somewhat delayed.

Then extensive testing and analysis of multiple scale-up conditions for VAL9 cells in GMP compliant culturing systems including media, matrixes and cytokines from P6 for maintaining human embryonic stem cells have been compared by P5 and thoroughly analyzed for its ability to maintain pluripotency of VAL9-hESC after long term-cultures (20 passages) and with stable karyotype.

To summarise the cGMP culturing systems DEF-XF (including cGMP coating) and DEF-XF (property of NovoNordisk) with gelatine/FBS as coating worked as good as the non GMP DEF-CS system a proprietary medium of Partner 5, in propagating VAL9.
In vivo teratoma assays were performed by P3 with VAL9 cells (provided by Partner 5) cultured using conditions 1-4 (below) in SCID mice following unilateral intratesticular (IT) injection of 1x10^6 cells (3 mice per cell line):
1) “VAL9” cells cultured Vitronectin / E8 (UCAM)
2) “VAL9” cells cultured GelFBS / E8 (Cellectis)
3) VAL9 cultured GelFBS / DEFXF (Cellectis)
4) VAL CIPF (positive control)
5) Vehicle Sham (negative control)

(END=Endoderm, MES=Mesoderm, ECT=Ectoderm)

#	Cell	Source	Culture Conditions			Tumours			
			Substrate	Media	Dissociation	#	END	MES	ECT
1	"VAL9"	UCAM	Vitronectin	E8	EDTA	3/3	Y	Y	Y
2	"VAL9"	Cellectis	GelFBS	E8	EDTA	3/3	Y	Y	Y
3	VAL9	Cellectis	GelFBS	DEFXF	TrypLE	3/3	Y	Y	N
4	VAL9	CIPF	Matrigel	CM	EDTA	1/1	Y	Y	Y
5	Sham					0/2	-	-	-

Table summarizing the results of the Teratoma analysis indicating the number of animals that formed tumours (red) and a breakdown of the pathologists analysis of the germ layers found within samples (Y = yes, N= no)

Conclusions
In vivo teratoma data show that cells expanded in E8 media performed better than those expanded in DEF-XF with regard their ability to form teratomas in the SCID mouse IT model suggesting **E8 is better** at maintaining the pluripotency of the cells, when measured using the gold-standard assay. However, important differences in cell delivery mean that we cannot rule out DEF-XF as a viable expansion media for this application, or conclude that E8 is the optimal media for future applications. The differences could be due to either:

- A genuine loss of pluripotency due to the culture method, or
- Difference in dissociation method used prior to injecting the cells(single cells v EDTA scraped as used in the E8 conditions tested)

Based on P2 results it was decided by Partner 6 to follow up on an E8-like medium for the expansion of VAL9 within the *InnovaLiv* project. Therefore the medium formulation was transferred to P6 CMO and a prototype medium batch was produced with qualified raw materials in a GMP facility. The majority of the quality control assays performed by the CMO (sterility, pH, osmolality, endotoxin and mycoplasma testing) have been done according to Ph. Eur. With this prototype medium, long term expansion studies using recombinant Vitronectin XF (Primorigen) as coating material were performed to evaluate whether hPSC will retain a normal karyotype under the given culture conditions. The hESC line SA181 as well as the hiPSC ChiPSC18 could be expanded for more than 30 passages with a mean population doubling time of 26 hours. They retained the typical morphology and a normal karyotype. Flow cytometric and immunofluorescence analysis for pluripotency and proliferation markers revealed that pluripotency and proliferation capacity were maintained over a period of at least 20 passages The medium has been made available and tested successfully by P3. The preclinical product will be launched to the market first half of 2015.

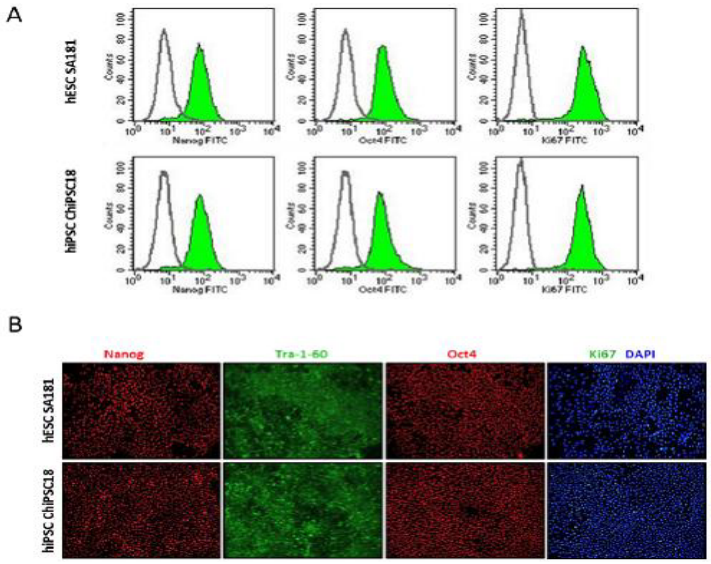

Figure: Assessment of pluripotency after 20 passages in CGX-E8-like expansion medium on Vitronectin XF. **A.** Flow cytometry for the pluripotency markers Nanog and Oct4 and the proliferation marker Ki67. Grey histogram isotype control, green histogram indicated antibody. **B.** Immunofluorescence analysis for the pluripotency markers Nanog, Tra-1-60, and Oct4, the proliferation marker Ki67 and the DNA marker DAPI.

3. Development of GMP-compatible cytokines

3.1 Activin A

The production process for Activin A has been validated as well as all analytical methods to ensure the consistent quality of the recombinant product. Stability data have been generated to verify a shelf life of at least 2 years. The production process from E. coli bacteria is free of animal-derived components and fulfills our specifications for a GMP grade product. The GMP grade product was launched to market in M23.

3.2 Oncostatin M

The process development and optimization for Oncostatin M production was concluded. The specific activity was determined by its ability to induce the proliferation of TF-1 cells using the international WHO-standard NIBSC 93/564 as reference. The production process from E. coli bacteria is free of animal-derived components and fulfills our specifications for a GMP compliant product (shares the same expression system and the main production process with GMP grade, except manufacturing process and analytical methods have not been validated, stability studies have not been concluded). The GMP compliant product was launched to market M31. Depending on the market demand, a GMP grade product will be launched to market in the future.

3.3 Bone Morphogenic Protein-4 (BMP-4)

The cytokine was produced in *E. coli* bacteria, and the production process development was well progressed. The process yielded reasonable amounts of biologically active BMP-4. However, crucial for stability and full activity was the use of a bovine-derived detergent, CHAPS. So far no

CHAPS with a certified safe origin and validated removal of adventitious viruses could be identified. Synthetic substitute detergents, as used for Activin A, caused a loss of activity. In addition, the protocol we finally decided on does not ask for BMP-4. For these reasons the project activities were stopped M24.

3.4 Hepatocyte Growth Factor (HGF)

As HGF is a complex heterodimeric protein that is produced by a human amniocyte cell line (CAP®), particular challenges arise for the downstream purification process:

- As HGF is produced as an unprocessed single chain, the site-specific cleavage has to be performed *in vitro*. This has been achieved in very high yield (>90%) in small scale experiments with two alternative proteases (Hepsin, Matriptase).
- Although the parental Master Cell Bank of the CAP® cells was excessively characterized for human and other adventitious viruses, we intend to include two potent virus removal/inactivation steps in the purification scheme.

The current small scale production process fulfils the specification regarding integrity, purity and activity. Production materials have been procured and qualified. However, to increase the yield of biological active material the upstream process needs to be optimized. The GMP compliant product is planned to be launched to market in March 2015. Depending on the market demand, we will decide on the further development of a GMP grade product.

In summary, all GMP compliant reagents and media have been developed by P6, except for one cytokine which will be finalized soon.

4. Differentiation of VAL9

Two differentiation protocols (one from Partner 2 and one from Partner 5) were combined with two GMP compliant culturing system, DEF-XF (developed by Partner 5) and E8 (commercial available) to differentiate VAL9-hESC to hepatocytes. Experiments were performed on VAL9 cells from two different sources “UCAM-VAL9” hESC adapted to E8 culturing system and “Cellectis VAL9” hESCs for comparative studies by P5.

All together the data generated by P5 (Cellectis) indicates that its protocol is superior to UCAM´s protocol to differentiate VAL9-hESC to hepatocytes even though of an immature phenotype

The next step was to further characterize UCAM-VAL9 hepatocytes cultured in E8-Vitronectin and differentiated using Cellectis´ protocol. The VAL9 hepatocytes express functional UGT1A1 (data from P4). UCAM-VAL9-hepatocytes were positive by the hepatic marker ASPGR-1.

While experiments in vivo (described below) were ongoing, it appeared that the HLA genotype of UCAM-VAL9 hESC did not match the HLA genotype reported in the documentation of the Spanish VAL9 originating from Spain’s National Bank of Stem Cell Lines (BNLC). The mismatch was confirmed. It was concluded that the “UCAM-VAL9” hESC was not of the same origin as the original Spanish VAL9-line.

The *InnovaLiv* consortium decided to thaw an original vial of VAL9-hESC of low passages, originating from the Spanish stem cell bank (BNLC) directly into the E8 culturing system and scale up for further differentiation to hepatocytes using Cellectis´ protocol. The original VAL9 was successfully thawed into the E8 culturing system and propagated without remarks for 20 passages with stable karyotype. However, the Spanish VAL9-hESC of low passages in E8 culturing system could not be differentiated to hepatocytes using Cellectis´ protocol. Therefore the combination DEF-XF culturing system with Partner 5´s protocol was chosen to continue work on.

5. Safety testing of differentiated UCAM “VAL9” hepatocytes using two in vivo assays of tumorigenesis

In order to gage the safety of cells expanded and differentiated using “GMP-suitable” scale up and differentiation conditions P3 assessed the ability of differentiated hESC derived hepatocytes to form tumours/teratomas in two different mouse models: (1) SQ injection of BALB/C nude mice to test for teratoma formation and (2) intrasplenic injection of SCID mice to test for possible engraftment and metastasis to liver and lung.

UCAM “VAL9” cells adapted to the E8 culturing system were differentiated to hepatocytes by Partner 5 using “GMP-suitable” conditions (Cellectis protocol, COAT1 as matrix), then shipped live to the CIPF (P3) where they were dissociated using EDTA/Accumax (according to the protocol provided by Partner 5) and injected into BALB/C Nude and SCID mice at a time corresponding to day 21 of the Cellectis differentiation protocol.

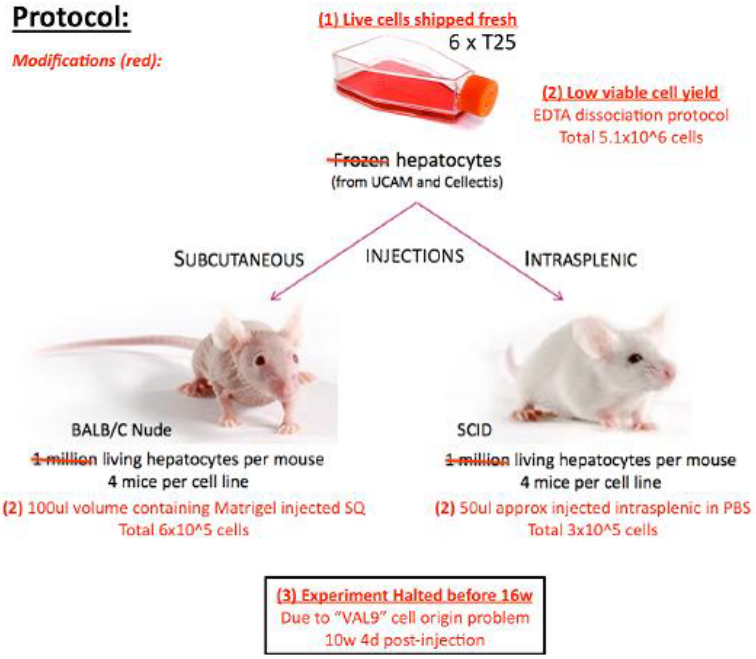

Figure: Schematic overview of the experimental design used to test the safety of GMP-suitable UCAM “VAL9” Hepatocytes.

Tumors were observed in 3 of 4 (75%) BALB/C Nude mice injected SQ with UCAM “VAL9” hepatocytes

When the above results were presented at the Innovaliv steering meeting (4th June 2014) two important issues were raised that could explain the tumour forming potential of the UCAM “VAL9” hepatocytes:

(1) The origin of the UCAM “VAL9” cells used to generate hepotocytes by Partner 5 and HLA data demonstrating that the cells were not VAL9 cells (see “problems” 2).
(2) The use of the GSK3β inhibitor “BIO” in the 7 day hepatocyte maturation phase

6. Safety testing of differentiated VAL9 hepatocytes differentiated in presence/absence of GSK3β inhibitor (BIO) using two in vivo assays of tumorigenesis

In the last step of the master protocol one of the components is an GSK3beta inhibitor (BIO) which has proven to maintain hepatic morphology at day 28 of differentiation and to have a beneficial effect on Phase I and II function in hPSC-derived hepatocytes and preventing overgrowth of the hepatic cultures of other cell types, while at the same time is inhibiting albumin expression of hPSC-derived hepatocytes (unpublished data from TBE). However, GSK3 beta inhibitor (BIO) is also known to be an activator of the Wnt-signaling pathway, which is known to play a role in tumorigenesis.

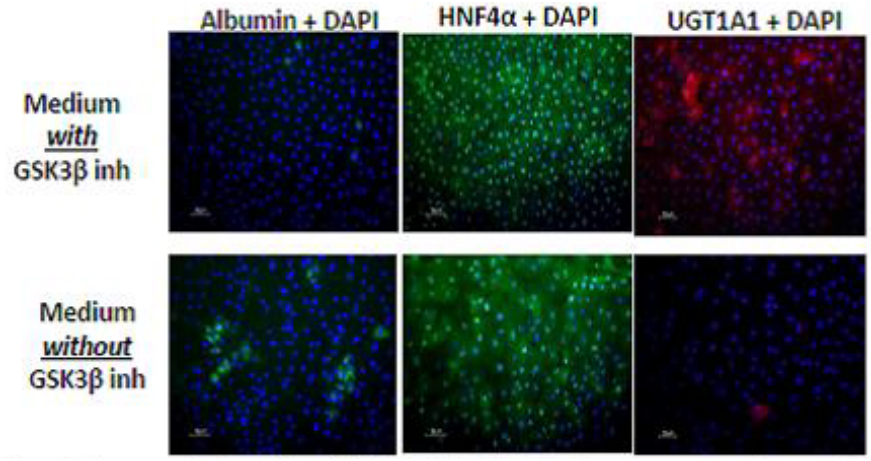

Figure: Immunocytochemistry of VAL9-hESC cultured in the presence or absence of GSK3beta inhibitor (BIO) day 27 of differentiation.

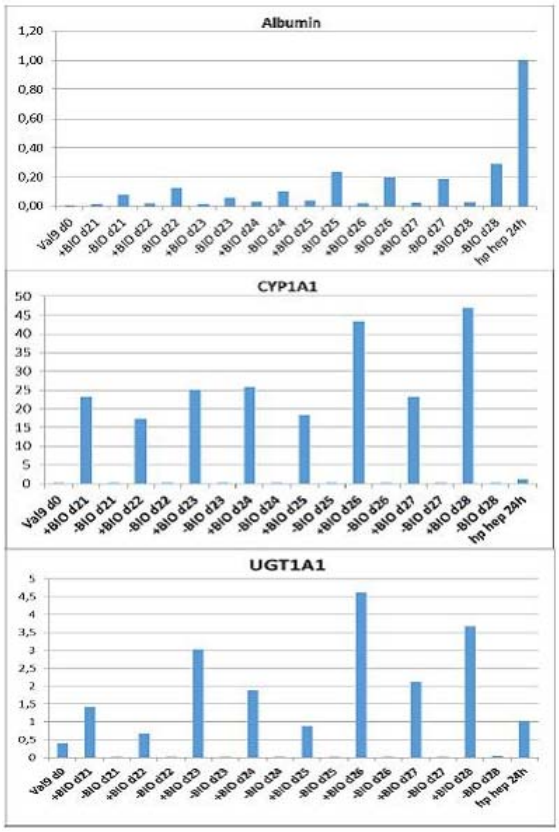

Figure: Gene expression analysis of ALB, CYP1A1 (phase I enzyme) and UGT1A1 (phase II enzyme) in VAL9-hepatocytes cultured in medium +/-BIO (GSK3beta inhibitor).

The two sets of VAL9-hepatocytes at day 18 of differentiation were shipped to Partner 3 for transplantation after a few days of recovery (d21 of differentiation) into mice and the cells´ tumorigenetic potential was assessed.
In addition to the usual positive (undifferentiated VAL9) and negative controls (PBS), VAL9 hepatocytes treated with (Group A) or without BIO (Group B) for the final 7 days of the differentiation protocol were injected.
Mice were lastly sacrificed 16 weeks following injection (January 2015).
No tumor was observed in both conditions subcutaneously or in the spleen, confirming that
HLA verified VAL9 hepatocytes expanded and differentiated in GMP-suitable conditions using BIO are “safe to use” according to *in vivo*. Further analysis is pending.

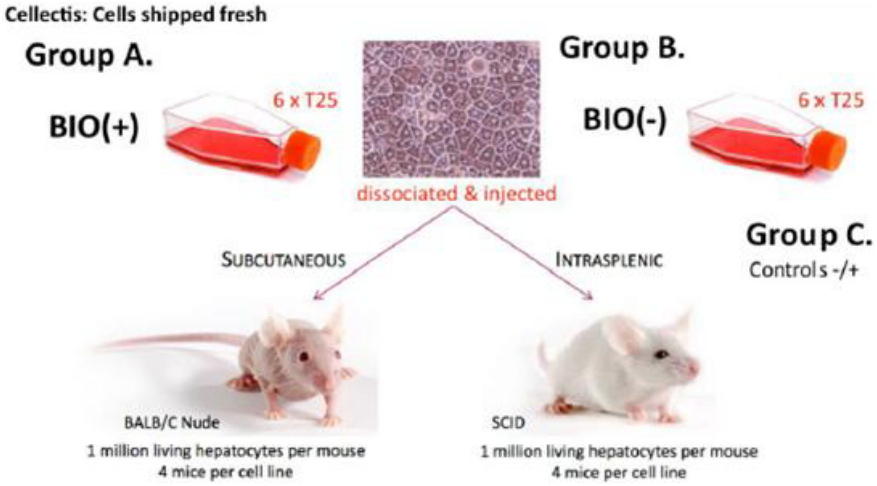

Figure: Schematic describing the experimental plan used to test the safety of VAL9 heptocytes generated using the GMP-suitable scale-up and differentiation protocols +/- BIO.

Nude mouse model: injected SQ
Group A (+BIO) - 4x animals: NO obvious SQ tumour tissue in injected flank
Group B (-BIO) - 4x animals: NO obvious SQ tumour tissue in injected flank

SCID mouse model: injected intrasplenically
Group A (+BIO) - 4x animals: NO obvious macroscopic phenotype
Group B (-BIO) - 3x animals: NO obvious macroscopic phenotype (one animal died shortly after the injection)
It was concluded that HLA verified VAL9 hepatocytes expanded and differentiated in GMP-suitable conditions using BIO are “safe to use” according to these *in vivo* preliminary analysis.

7. Other differentiation protocols

Academic partners (P1, 2, 4) have developed protocols to direct differentiation of hPSCs into a near homogenous population of foetal like hepatocytes which can undergo functional maturation following prolonged culture. The procedure uses chemically defined media (CDM) devoid of serum and complex extra-cellular matrices such as Matrigel. The protocol comprises four stages, which mimic the embryonic development of the liver and enable the production of differentiated cells following a natural path of development.

7.1 Partners 1A and 4

Partner 1A defined a multistep protocol using xenofree GMP-compatible conditions to differentiate VAL9 into hepatocytes, using cytokines and gelatin provided by Partner 6 and assessed their functionality in vitro and in vivo with P4.

In vitro
VAL9 expressed specific markers of differentiated hepatocytes such as albumin and alpha 1 antitrypsin, CYP3A4 and 85% of hepatocytes expressed Asialoglycoprotein Receptor as assessed by flow cytometry.

Moreover, VAL9-hepatocytes displayed activity for UGT1A1 and for several CYP. They also excreted urea.

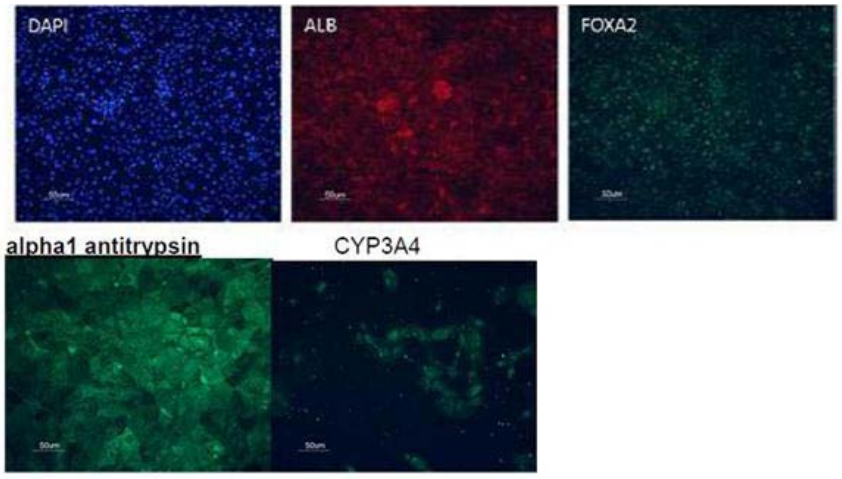

Figure Expression of hepatocytic markers

In vivo

VAL9-hepatocytes have also been validated in vivo for cell functions for safety issues. GFP transduced cells engrafted and were able to proliferate in a mouse model of acute liver failure and to rescue transplanted mice. Engrafted human cells represented up 10% of the liver and this result represents the first report that such a repopulation is achieved in a model with acute failure.

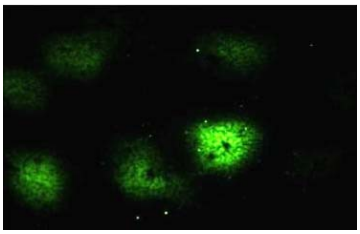

Figure: Val-9 hepatocytes were found into the liver of animals with acute liver failure: large clusters of GFP-VAL9 hepatocytes, lentivirally transduced prior transplantation represent cell proliferation in situ.

Histology analyses showed no evidence of tumorigenicity in transplanted livers and no cell dissemination was observed in other organs analysed such as lung (*Laia Tolosa et al, in preparation*)

7.2 Partner 2

P2 has focused on validation of different GMP reagents to develop a GMP protocol for generating hIPSCs derived hepatocytes to create an open access method publically available for other to use based on our previous published protocol. For that, VAL9 grown in E8 conditions for 30 passages were systematically differentiated in diverse combination of GMP reagents. The procedure uses chemically defined media (CDM) devoid of serum and complex extra-cellular matrices such as Matrigel. The protocol comprises four stages, which mimic the embryonic development of the liver and enable the production of differentiated cells following a natural path of development. Each stage is marked by the expression of specific genes while the hepatocytes like cells obtained after 25 days of differentiation display functional characteristics shared by both foetal and adult hepatocytes including Albumin and A1AT secretion, Glycogen storage, Cholesterol up take, and inducible detoxification activity of cytochrome P450 enzymes including CYP3A7, CYP1A1. As maturation is continued foetal characteristics begin to diminish while functions associated with adult functionality increase to reach a maximum level at Day 35.

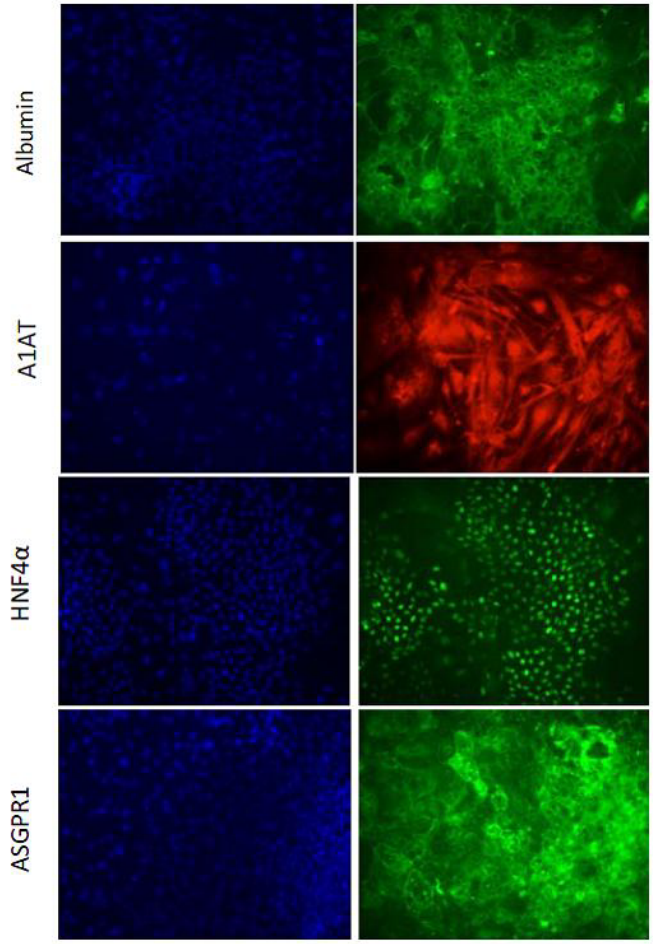

Figure: Immunostaining showing homogenous expression of hepatic markers in hPSCs differentiated for 3 days in cGMP protocol

After 40 runs of differentiation, P2 has been able to establish an entire list of reagents, which are cGMP or GMP. VAL9 were then differentiated 3 times using the entire GMP protocol. Preliminary results suggest that this GMP version of UCM protocol generates hepatocytes resembling cells generated with non-GMP protocol. These conditions allow also differentiation of other pluripotent stem cells. After 40 run of differentiation, P2 has been able to establish an entire list of reagents, which are cGMP or GMP. VAL9 were then differentiated 3 times using the entire GMP protocol. Preliminary results suggest that this GMP version of UCM protocol generate hepatocytes resembling cells generated with non-GMP protocol. These conditions allow also differentiation of other pluripotent stem cells.

7.3 Partner 6 (Cellgenix) developed differentiation media using safe and pure raw materials which can easily be upgraded to GMP grade.

Based on the master protocol developed by P5, P6 has developed GMP reagents and optimized the culturing condition to allow the efficient differentiation of hPSC-lines, both hESC and hiPSC-lines (from P5) to hepatocytes. Since it is not allowed to work with VAL9-hESC-line, reagents and media of GMP compliant were developed using SA181 hESC and iPSC lines and distributed to Partner 3 and 5 for verification of its ability to differentiate VAL9-hESC to hepatocytes. The prototype master protocol was used as a reference. VAL9-hESC could be differentiated into hepatocytes using Cellgenix GMP reagents. At day 18 of differentiation hepatocytes had been formed in both conditions and GMP compliant Fibronectin from Akron can substitute coat1 used in the master differentiation protocol.

Analysis of gene and protein expression, and functional phase I and phase II enzymes show similar characteristics of the GMP compliant and prototype VAL9-derived hepatocytes revealed no

difference. Method validation, confirmation of consistency and stability studies are required for final approval of the GMP grade of the media.

7.4 VAL9-hepatocytes functions

Partner 4 (HULAFE) has focused on the characterization of hepatocytes derived from pluripotent stem cells. Measurements of CYP activities are critical to determine the degree of differentiation achieved in hepatocytes differentiated from pluripotent cells and xenobiotic metabolism due to CYP enzymes is probably some of the most “hepatic-specific” functions.

HULAFE has developed an UPLC/MS platform to determine the capabilities of the cells to metabolize specific cytochrome P450 (CYP) substrates. Each substrate is metabolized by a single CYP in the adult hepatocyte, thus assessing by a unique analysis the level of functionality of the enzyme. In fact, the profile of activities evaluated in our assay is hardly present in newborn hepatocytes and is only acquired several months after delivery. In summary, measurements of CYP activities are critical to determine the degree of differentiation achieved in hepatocytes differentiated from pluripotent cells. Cells are incubated for 24 hours in cell media containing 8 substrates that are metabolized by a specific CYP.

Table 1.1. Selected substrates for CYP450 activities measurement

CYP (adult)	Substrate	Metabolite	Activity
1A2	Phenacetin	Acetominophen	Phenacetion O-deethylase (POD)
2A6	Coumarin	7-HO-Coumarin	Coumarin-7-hydroxylase
2B6	Bupropion	HO-Bupropion	Bupropion-hydroxylase
2C19	Mephenytoin	4´-HO-Mephenytoin	Mephenytoin-4´-hydroxylase
2C9	Diclofenac	4´-HO-Diclofenac	Diclofenac-4´-hydroxylase
2D6	Bufuralol	Hydroxybufuralol	Bufuralol-hydroxylase
2E1	Chlorzoxazone	6-HO-Chlorzoxazone	Chlorzoxazone-6-hydroxylase
3A4	Midazolam	1´-HO-Midazolam	Midazolam-1´-hydroxylase

After the incubation time, supernatant is obtained and analyzed by a single injection by UPLC/MS.

With this platform P4 determined CYP activity in pmol/min/mg of protein for Partner 1A, P2 and P5. To obtain a clear reference about the degree of differentiation of the cells, CYP activity was compared with the average from more than 40 different primary cultured hepatocytes or to neonates hepatocytes.

UGT1A1 activity in VAL9-hepatocytes was also assessed for the different differentiated cells as well as urea secretion.

7.5 Cryopreservation

P5 has developed a non-GMP protocol for cryopreserving hiPSC derived hepatocytes that retain its hepatic morphology after thawing and express hepatic markers such as CK18 and AAT, ten days post thawing. In addition Cytochrome P450 function is retained for CYP families 3A and 1A.

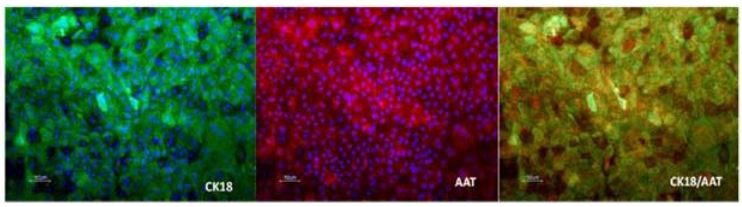

Figure: Immunocytochemistry of the hepatic markers cytokeratin 18 (CK18) in green and alpha 1 antitrypsin (AAT) in red performed on frozen hiPSC-HEP 10 days post thawing.

Cryopreservation of VAL9-hepatocytes (and hepatic progenitors) (P4, P1A) using GMP-grade freezing medium, UW-solution, was successfully performed with retained hepatic morphology and expression of hepatic markers after thawing. The technology is ready for tech transfer to GMP compliant VAL9-hepatocytes once all GMP compliant reagents are available.

Workpackage 2 focused on *Production of tools for hiPSC-based therapy of genetic liver diseases*

The first step was to obtain all the authorizations for getting patients' biopsies and then to reprogram control normal fibroblasts together with patients' fibroblasts.

1. Reprogramming of normal foreskin fibroblasts (CRL 2097 from ATCC)

1.1 Retroviral reprogramming

Partner 1A reprogrammed commercial normal fibroblasts with a polycistronic retroviral vector (rRV-OCT4-SOX2-KLF4-VMYC) purchased from Vectalys (Labège, France) encoding human OCT4, SOX2, KLF4 and MYC. Amongst several iPSC-like clones two have been characterized for pluripotency in vitro and in vivo in immunodeficient mice in which they generated tumors as assessed by Partner 3 (CIPF).

1.2 Messenger RNA-mediated reprogramming

Integration of retroviral vectors results in insertional mutagenesis that could have a detrimental effect on proliferation and/or differentiation of the iPSCs. In contrast, reprogramming by transfection of mRNAs leaves no traces in the genome thus avoids insertional mutagenesis. Partner 1A defined conditions to prepare recombinant mRNAs for cell reprogramming (Oct3/4, Sox2, Klf4, c-Myc, Lin28) and to generate 2 series of iPSC clones by using iterative mRNA transfections. iPSC clones all expressed pluripotency markers and displayed a normal karyotype. These cells gave rise to teratomas that exhibit tissues from all three germ-layers (Partner 3).

1.3 Genomic integrity of iPSC clones

Despite the significant potential applications of hiPSCs, the genomic integrity of these cells still raises some concern.

iPSC genomic integrity was studied in comparison with that of primary fibroblast cell population and iPSC clones derived from the same primary fibroblast cell population by polycistronic lentiviral vector-reprogramming. using Genome-Wide Human SNP Array 6.0 from Affymetrix.

It was observed 3 times less SNV (Single Nucleotide variation), in comparison to primary fibroblasts, in the iPSC lines that have been reprogrammed with mRNA vs. iPSC lines reprogrammed with the lentiviral vector. Concerning the CNV (Copy number Variations), there was no significant difference between both types of cell lines in the number of deletions, in contrast, the number of duplications is 3 times less in mRNA-derived iPSC lines compared to virally derived ones. (***Steichen et al, Stem Cells Transl Med 2014; 3(6):686-91)***

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In addition a hiPSC line, generated by transfections of messenger RNAs displayed several genomic abnormalities. Importantly, the genomic rearrangements do not impact the iPSC line characteristics in terms of stemness-marker expression but it impairs the iPSC line ability to generate teratomas *in vivo*. Karyotype analysis showed a complex genomic rearrangement, which remained stable during long-term culture. SNP analysis revealed the presence of a large 1q region of uniparental disomy (UPD), demonstrating for the first time that UPD can occur, in a non-compensatory context, during non-integrative reprogramming of normal fibroblasts. This observation highlights the need for careful selection of hiPSC lines for the genomic integrity using all genetic analysis techniques, including SNP, which solely allows detection of loss of heterozygozity in an UPD context**.** (***Steichen et al, Stem Cells Transl Med 2015 E Pub ahead of print***).

2. Generation of iPSC clones from patients with metabolic diseases

2.1 Reprogrammation of FH patients' fibroblasts

Two patient's fibroblasts isolated from biopsies were transduced with lentiviral polycistronic reprogramming vectors. Nine clones have been amplified and cryopreserved (5 with PG 1 and 6 with FH 2).

- PG 1 male, born *13.05.1985* Homoz D283Y
- FH 2 male, born *18.08.1987* Homoz *mutation to be confirmed*

- The sequencing of the region bearing the mutations confirmed that FH is homozygous for the mutation A519T : GCC > ACC. By contrast PG was found heterozygous for 2 mutations: –D283Y (GAC-> TAC)–T705I (ACC->ATC)

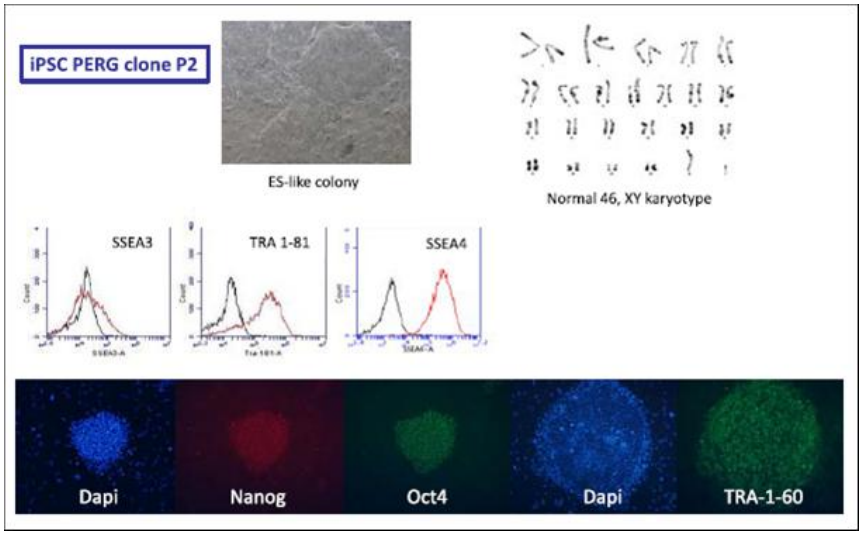


Figure: An exemple of analysis of pluripotency criteria in one iPSC line

- **CE-3** A third patient' biopsy was obtained and fibroblast reprogramming performed with episomes (a non integrating approach). This patient is homozygous for *LDLR* mutation: Q12X which introduces a stop codon close to the N terminus of the protein resulting in an absence of protein.

The presence of this mutation has been verified on the fibroblast DNA and one several clones of derived iPSCs characterized for their pluripotency.

2.2 Reprogrammation of fibroblasts from a patient with Haemophilia B

From a patient with a mutation of Factor IX gene : E387K (G → A) mutation, reprogrammation gave rise an iPSC line called iPSC HB2poly2 whichnhas been fully characterized for pluripotency.
Three other patients' samples were obtained recently but no iPSC clones up to now could be generated either by integrative or by RNA reprogramming approach for unknown reasons.

2.3 Reprogrammation of fibroblast patients with Crigler-Najjar type I (CN)

Partner INSERM 1B first received from UCAM partner an iPSC cell line generated from a CN patient using retroviral vectors. This CN cell line was differentiated into hepatocytes. Two french patients were then identified:

- Patient CN1-01: 25 years-old woman, homozygous for c.865-1G>A (Servedio et al., 2005), a mutation of site splicing acceptor of intron 1-2 leading to an instable mRNA
- Patient CN1-02: 35 years-old woman, homozygous for c.923G>A (Erps et al., 1994), a missense mutation in exon 2 leading to a functional protein.

Primary fibroblasts from these two patients were isolated and expanded in GMP conditions in the Cell and Gene Therapy Unit (UTCG) in Nantes from a small skin biopsy during September (patient CN1-01) and in October 2012 (patient CN-02). They were cryo-preserved at passage 3: 40 vials for patient CN1-01 (75 million cells) and 26 vials for patient CN1-02.

These cells were sent to P2 (UCAM) who performed different assays but was unsuccessfull to generate iPSC clones for technical problems.

In parallel to skin biopsies, blood samples (100 ml) were harvested from the patients in order to isolate circulating Endothelial Progenitor Cells (EPC) and EPCs were successfully isolated only from Patient CN1-01.

The hIPSC lines generated were expanded by P1A and 1B (FH/HB/CN) and P2 (CN). Around 50 vials were produced for each line using defined culture conditions and have been banked in each respective group.

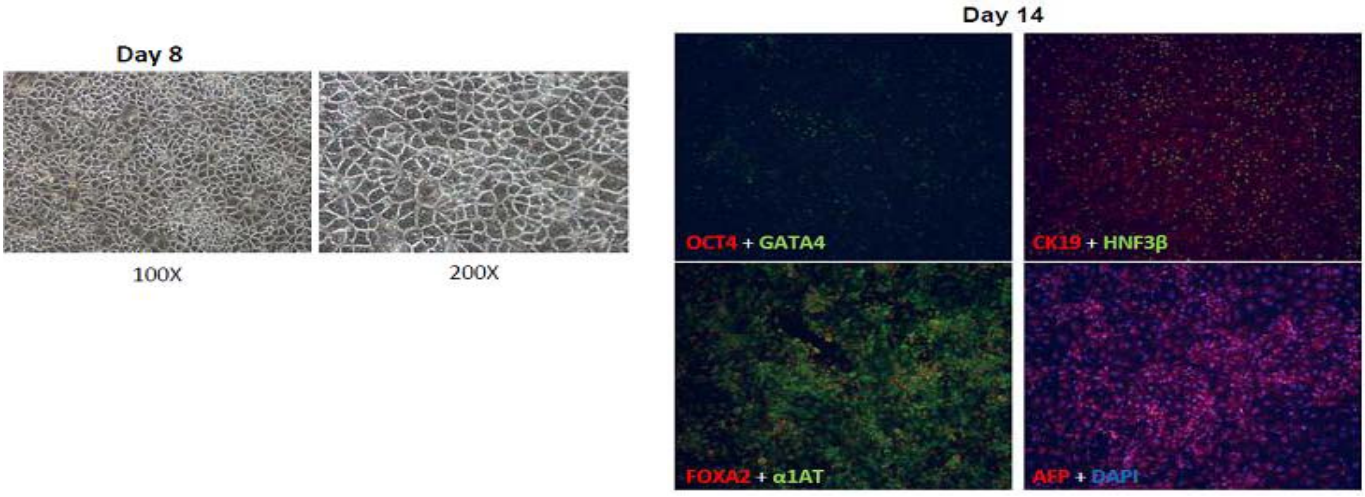
Partner 4 has also generated fibroblasts from patients with diverse diseases in GMP conditions.

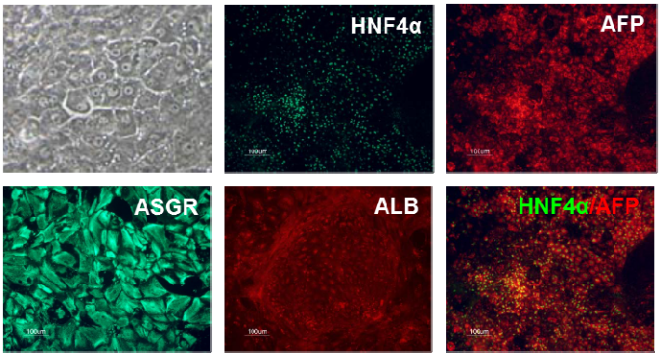
3. Differentiation studies

Conditions to differentiate the different iPSC lines into hepatocytes were defined for each disease. Examples of differentiation are illustrated below

3.1 HB iPSCs

Between d8 and D14 of differentiation, expression of pluripotency markers no longer persist and hepatoblasts markers are expressed, as shown below.

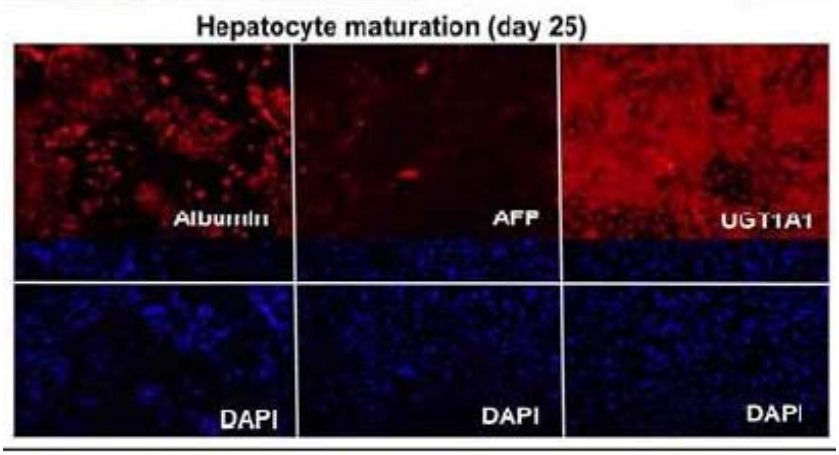




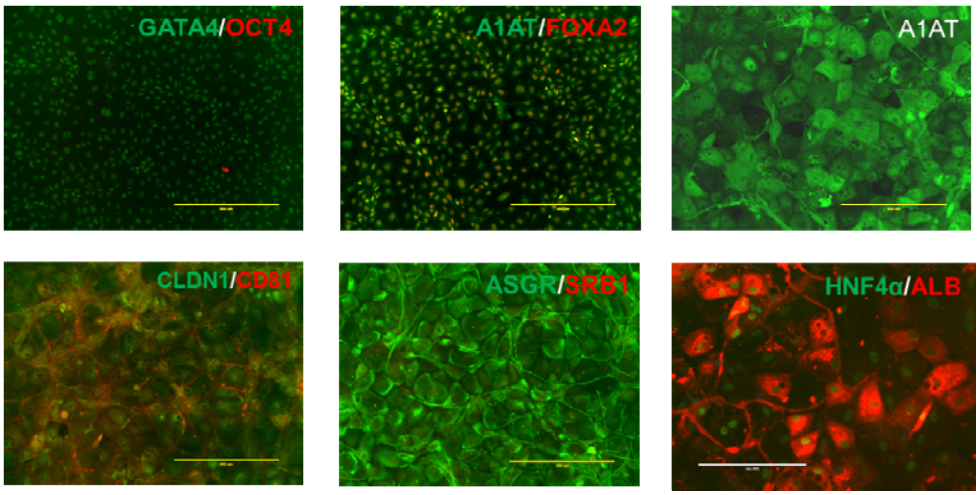
Then hepatoblasts were differentiated further into more differentiated hepatocytes like cells from FH and CN patients, as shown above.

3.2 CN iPSCs

The protocol defined resulted in expression of Albumin and UGT1A1 in CN-hepatocytes at day 25 of differentiation and a concomitant decrease of AFP expression.



3.3 FH-iPSC



CE3-hepatocytes at day 27 no longer expressed OCT4 but express specific hepatocyte markers: FOXA2, HNF4α, A1AT, ASGR, CLDN1, CD81, SRB1 and ALB. Interestingly these iPSC-hepatocytes derived from a patient devoid of LDLR protein (a coreceptor for HBC entry) expressed the main proteins involved in HCV virus entry (claudin, CD81 and SRB1).

4. Correction of genetic defects

4.1 Gene correction in hiPSCs using gene therapy approach.

Partner 1A defined conditions for iPSC and iPSC-hepatoblasts transduction using lentiviral vectors encoding GFP under the transcriptional control of either EF1α or hepatic-specific ApoA-II promoters. Transduction efficiency was approximately of 50%.

FH-hepatoblasts were then transduced with APOA2-GFP lentivector as control or APOA2-LDLR.

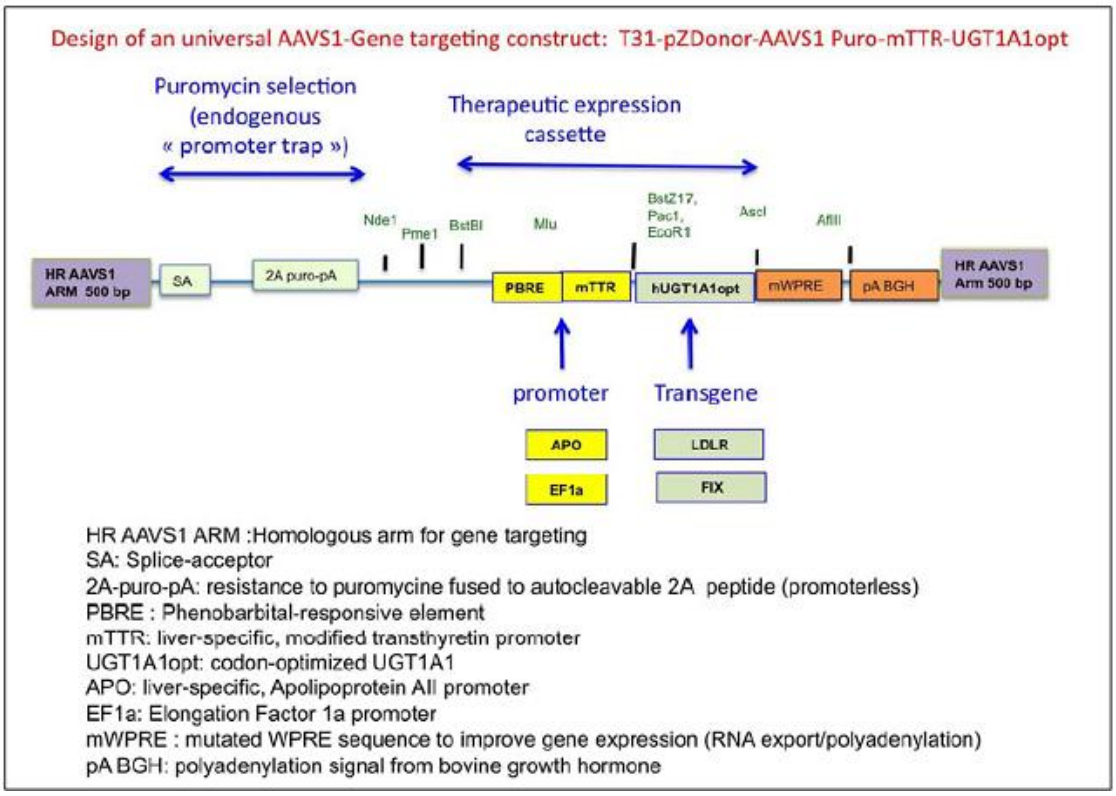
Western blot analysis of transduced cells showed that transduction of LDLR vector resulted in expression of the transduced protein, suggesting the functionality of this approach.

4.2 Correction of genetic defects by targeted transgene addition

Advent of a new technology based on Zinc Finger Nucleases (ZFNs) has been shown to greatly increase the efficiency of gene targeting in human pluripotent stem cells and to bypass the limitations associated with conventional homologous recombination strategy. Partner 2 has validated the use ZFN/TALENs to insert transgene into the adeno-associated virus type 2 (AAV2) target region on chromosome 19, known as the AAVS. This locus is devoid of genes and has been shown to allow transgene expression and has been previously been validated for gene therapy. To validate this approach, P1B developed its ZFN and then used a donor/targeting vector containing the GFP gene under the control of the ubiquitous CAGG promoter. hiPSCs were co-transfected with the ZFN pair and this donor vector. After selection the cells which had stably integrated the transgene remained pluripotent, could be differentiated into the 3 primary germ layers and expressed GFP, confirming that Knock In transgene in the AAVS-1 locus using ZFN represent an efficient method to express report gene ubiquitously

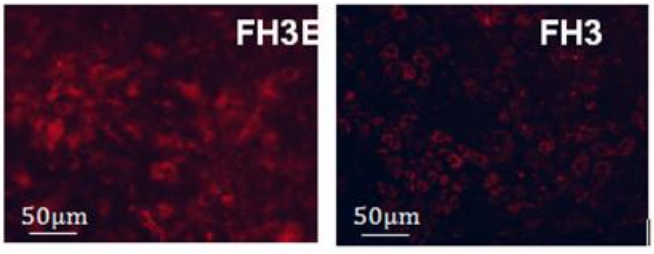
P1A and 1B constructed the gene targeting construct expressing UGT1A1, RLDL and FIX cDNAs respectively and performed different tests to validate the constructs.

Targeted integration was also achieved for CN-iPSC with Transcription Activator Like Effectors Nuclease chimeras (TALENs),



In conclusion correction of genetic defects by targeted transgene addition was successfully achieved in CN, FH and HB iPSCs at the molecular level in different clones for each disease.

Moreover, the phenotypic correction of corrected clone FH-C3E was demonstrated by internalization of DiL-LDL, a fluorescent ligand of the receptor into FH3E compared to non-corrected FH3-hepatocytes



4.3 Development of alternative method of correction of Inherited Metabolic Diseases in hIPSCs

We also developed alternative strategy including the use of another safe harbor locus and also the development of homologous recombination approach using CRISPR/Cas9 technology. Finally, we develop a conditional system of expression, which could enable gene therapy in a time specific manner.

WP3 – Assessment of engraftment capacity and functionality of hiPSC

The functionality and safety of hiPSC-derived hepatocytes has to be tested *in vivo* in different animal models to assess their engraftment and proliferation capabilities, as well as to confirm the metabolic competence of iPSC-derived hepatocytes. Also correction at the genomic level of diseased clones should restore hepatic function in vivo;

1. Transplantation of corrected FH-iPSC-derived hepatocytes into Rag2-/-gamma c-/- mice and of corrected HB-iPSC-derived hepatocytes into F9 KO mice

The generation of patient-specific iPSCs was significantly delayed for all the diseases 10 months for Familial Hypercholesterolemia and Hemophilia B. The conditions for robust differentiation of the iPSCs into hepatocytes was time-consuming and this step was necessary prior to select the iPSC clones to be genetically corrected.

1.1 Deviation plan

The work initiated in the previous European project LIV-ES was continued. Since fetal hepatic progenitors are bipotential and have the ability to give rise to fetal hepatocytes but also to fetal cholangiocytes (biliary cells), we wished to demonstrate the robustness of our protocol to generate hepatoblasts. We demonstrated that hepatoblasts generated from human H9 ES cells but also from human iPSCs could be differentiated into cholangiocyte-like cells, bearing primary cilia, and displaying specific proteins important for cholangiocyte functions. The conditions are being adapted to VAL9 cells. (***Dianat N et al, Hepatology 2014, 60:700-714***.)

1.2 Improve iPSC-hepatocytes engraftment

A protocol to transplant safely primary hepatocytes after one or 2 transient partial portal embolizations was defined in control rats. This resulted in an increase in the proportion of engrafted cells. Transposition to Gunn rats is ongoing to transplant CN corrected iPSC-hepatocytes after embolization (INSERM 1B). This increase in cell engraftment should result in enhancement of biological activity and in bringing other convincing evidence of the safety of the cells (*manuscript in preparation*).

2. Correction of Gunn rat with corrected CN-hiPSC-derived hepatocyte transplantation

Before achieving this goal, the problem of avoiding the rejection of the xenograft of human cells by the rat had to be solved. This has been attempted by three approaches: i) induction of tolerance to human cell xenograft in the Gunn rat; ii) immunosuppression treatment; iii) engineering of an immunodeficient Gunn rat strain that will be a valuable animal model for testing the engraftment capacity and functional efficacy of human hepatocytes whatever their origin.

2.1. Induction of tolerance to human cell xenograft in the Gunn rat

Adenoviral vectors encoding the immunomodulatory molecules CTL4Ig (Ad-CTLA4Ig) and CD40Ig (Ad-CD40Ig) were developed by P1B and different doses infused to the Gunn rats but this approach failed to maintain human cells in the liver of transplanted animals.

2.2. Immunosuppression protocol in the Gunn rats

P4 has developed an immunosuppression protocol, which, after transplantation of neonatal human hepatocyte transplantation results in long-term correction of hyperbilirubinemia in Gunn rats (*manuscript in revision in Liver Transplantation*)

2.3. Transplantation of iPSC-hepatocytes into the Gunn rats

Using the same immunosuppression protocol, transplantation in adult Gunn rats resulted in long-lasting significant decrease in hyperbilirubinemia. Importantly, liver engraftment of hepatocytes differentiated from a normal iPSC line (provided by P2) was sustained for a long period (6 months) without signs of tumour formation (carcinoma or teratoma) and without signs of liver injury as assessed by histological and blood parameters analyses. In addition, no teratoma was observed 2 months after direct injection of HLCs in the testis, confirming the absence of residual undifferentiated hiPSC in the hepatocyte population (*manuscript in preparation*).

2.4 Generation of different Gunn and immunodeficient rat models

P1B has generated

- a UGT1A1-/- rat by invalidating UGT1A1 gene in Sprague-Dawley rat (the strain background of *IL2Rγ -/-* rats to *Rag-1 -/-* rats) using CRISPR-cas genome editing technology.
- a SCID Rag1$^{-/-}$ IL2Rγ$^{-/-}$ rat

Generation of UGT1A1-/- rat x SCID Rag1$^{-/-}$ IL2Rγ$^{-/-}$ rat is ongoing.

WP4 – Clinical use and Compliance with regulatory and ethical legislation

In general, to obtain the authorization of AGEMED for performing a clinical trial, the *quality of the drug, its safety and its expected clinical efficacy* has to be properly documented.

InnovaLiv is based on the differentiation of hESC to hepatocytes. The manipulations done on these cells before their clinical use are beyond the borders accepted in a conventional cell transplantation procedure. Consequently, *InnovaLiv* cells are considered a pharmaceutical for an *advanced cell therapy*. Hepatocytes will be derived from the VAL9 cell line which is deposited at the Spanish National Bank of Stem Cells. The partners formally applied for authorization to the pertinent Office (Comisión Nacional para la donación y utilización de células y tejidos humanos), and obtained the approval to derive hepatocytes from such cells.
Partner 4 (HULAFE) has had several working meetings with the Spanish regulatory agency (AGEMED) to establish the safety regulatory requirements knowing the nature and characteristics of the *"drug"* to be used, manufacturing procedures, and quality issues concerning the cell preparation (pre clinical dossier), purpose of the clinical trial and safety issues concerning the clinical application. AGEMED has provided some recommendation to prepare the pre-clinical dossier summarized below.

A) SAFETY AND QUALITY ASSESSMENTS

1. Manufacturing process:

Cell production

Human ES cells will be expanded in the selected GMP medium using a feeder free cell culturing technology and multi-layer flasks. The hepatocytes will be derived according to the differentiation protocols described above. SOPs will be generated and all components will be of GMP or close to GMP grade. Highly reproducible protocols will be used. The whole manufacturing process will be strictly monitored by the following QA/QC criteria:

- Sterility tested negative
- Mycoplasma and pathogens tested negative
- Characterisation markers for hepatocytes; e.g. HNF4α, α1-AT, albumin, PAS
- CYP-levels of certain relevant CYPs to the treatment of the patients in the clinical trial
- Absence of markers for undifferentiated cells (PCR for e.g. Oct4 and Nanog

Every batch of produced hepatocytes will be analysed for the QC parameters above. The stability of the process will be evaluated with morphological inspection, as well as using gene expression profiles at several check-points during culturing of hESC and during hepatic differentiation. For monitoring of the traceability Cellectis has strict QA/QC documentation and SOP systems in place for the already existing production of hepatocytes derived form hESC for in vitro use under GLP conditions.

Cell purity

The purity/homogeneity of the cell product will be assessed by morphological inspection as well as a defined set of gene and protein expression signatures, as assessed by any of the following techniques: QPCR, flow cytometry, immunohistochemistry, a combination of several of them.

2. Cell stability and genetic integrity

This part of the preclinical assessment is aimed to ensure that the cells do have genetic stability and integrity, so the risk for tumorigenicity or immunogenicity is minimized. Examples could be identifying specific chromosomal aberrations that tend to occur in stem cell culture and its consequences regarding safety. Limitations in the number of cell passages should be declared and monitored to reduce the likelihood of genetic alterations *in vitro*.

3. Dosing and pharmacokinetics:

Conventional pharmacology and toxicology studies generally do not apply to cell therapy in humans.

4. Biodistribution*:*

Animal data may be required in our case in order to know the distribution of the cells used in our cell therapy clinical trial. Whenever possible, a human-relevant animal model should have been used, as well the route of administration. Eventually, labeled cells (isotopic, genetic, magnetic, others like cytometry) may have to be used to demonstrate the distribution and or migration of cells from the site of application, to ensure that cells implanted in the liver do not escape to other tissues.

5. Immunogenicity and immunotoxicity:

The immunotoxicity of a clinical product is difficult to assess from studies typically conducted in immune-compromised or depressed animals. We assume that hESC-derived hepatocytes may not display a fully different surface antigen repertoire, but this needs to be tested.

6. Tumorigenicity

Most likely this is the risk event that the regulators will ask to be demonstrated convincingly and in detail. In his context it is important to ensure the *cell purity* understood as the homogeneous state of differentiation and cell lineage of each lot of differentiated cells.

Experiments conducted with nude mice in order to demonstrate each lot of hESC-derived hepatocytes is safe, will need to be performed.

***7. Cell functional activity*:**

To ensure that hESC derived hepatocytes metabolically perform like human hepatocytes is being assessed according to several criteria:

- Enzymatic activities typical of adult hepatocyte biotransformation (Cytochrome P450 and conjugating enzymes)
- Ureogenic capacity assessed by the conversion of ammonium in urea, indirectly ornithine transcarbamylase (OTC) activity
- Other metabolic activity parameters deemed appropriate (plasma protein synthesis, carbohydrate metabolism, etc.)

Regulatory and Ethical approval for a Pilot trial (compassionate use) of a Cell Product: The clinical dossier should contain the aim of the study (scope), the type of clinical trial, intended posology

(route of administration, number of cells, number of infusions), clinical/analytical parameters to assess the outcome of the cell therapy, long term follow up (adverse/unexpected effects, reporting system) and approval will be issued under well-defined criteria (not a *"broad, undefined"* approval will be issued). Paediatric patients with inherited hepatic metabolic diseases on follow up in our Unit.

Metabolic disease	Number of Patients	Present situation
Tyrosinemia I	6	Stable On NTBC
Wilson Disease	5	Pharmacologic Treatment Mild Liver Dysfunction
Glucogenosis	5	1 stable Post Hepatocyte Tx 4 Moderate Liver Dysfunction
Urea Cycle disorders	3	Stable Pharmacologic Treatment + Diet
Familial Hypercholesterolemia	2	1 Partial response to therapy 1 on Plasmapheresis
Peroxisomal Disease	2	1 stabile 1 Neurologic dysfunction worsening
Mitochondrial Disease	1	Neurologic dysfunction
Crigler Najjar type 1	1	Improvement Post Hepatocyte Tx . At present Bilirubin increasing

In summary, the SOPs for the manufacturing, quality control and safety assessment of the VAL9 and VAL9-derived hepatocytes have been developed and delivered. A first draft of the pre-clinical dossier has been agreed by the Consortium with AEMPS during the first reporting period. In the present draft, a tentative final version of the dossier has been defined and proposed to AEMPS, which includes cell quality and safety and efficacy assessments of the non GMP cells to be used in the clinical trial. Preliminary results from P1A and P4 indicate that transplanted VAL9-hepatocytes into a mouse model of acute liver failure are safe and functional *in vivo* helping to recover the hepatic functionality of the host liver without any sign of tumorigenicity or human cell dissemination to other organs.

The ultimate goal of this project was to demonstrate that cell therapy with hESC-derived hepatocytes can safely and efficiently restore metabolic functions, at least transiently, in diseased livers. Therefore, another task of WP4 was to define the criteria for selecting patients likely to benefit from the proposed cell-based therapy. Based on these criteria amongst paediatric patients with inherited hepatic metabolic diseases included in a follow-up in P4 unit, one patient with a chronic metabolic liver disease, Crigler-Najjar type 1, has at present been selected based on clinical features.

- The manufacturing site has been identified to the Sahlgrenska University Hospital, see authorization to use the SU facilities enclosed – certification from the Competent Authorities of the production site.
- Standard operational procedures (SOPs) for the manufacturing of GMP hESC-derived hepatocytes from VAL9 has been drafted and prepared for submission to the Swedish Medical Agency, Läkemedelsverket, according to the QA/QC criteria requested by the Spanish agency AGEMED for performance of the clinical trial.
- Criteria for release was discussed and identified in the pre-clinical dossier and initial discussions with a qualified person for approval of the final produc (GMP VAL 9-hepatocytes) were held.

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. Indeed, 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. Although the use of induced pluripotent stem cells (iPSCs) generated from somatic cells of patients with liver metabolic diseases offer new perspective for personalized medicine.

The *InnovaLiv* 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 even the economy of the European community.

By combining the expertise of academic partners in hESC biology and differentiation with SME leaders in large-scale technology and GMP manufacturing, the consortium has developed reproducible, animal-free GMP compliant conditions for the efficient differentiation of hESC to hepatocytes 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:
WP1 of the project has successfully identified protocols that can be used on a large-scale to maintain hESC cultures in the pluripotent state for long periods of time without increasing chromosomal instability as well as for pluripotent ES cell banking. Testing and verification of these methods was performed using VAL9, a hESC line developed in the EU (Spain) and also Swedish ES lines. Approaches to differentiate VAL9 and pluripotent stem cells into hepatocytes and cryopreserve these cells in GMP compliant conditions have been defined and differentiated cell safety and functionality validated in vivo. GMP-grade components such as growth factors and media for all stages, from pluripotency to hepatocytes, have been produced and validated by different partners. Accordingly a pre-clinical dossier has been developed which includes safety and quality control standards for the cell product to be used in a clinical trial.

Another critical objective of Innovaliv has been to produce new hiPSC lines from patients with metabolic diseases and to develop innovative tools to correct the inherited defects which impair hepatic function. These goals have focused on three inherited metabolic liver diseases: Familial Hypercholesterolemia (FH), Hemophilia B and Crigler-Najjar. These patient-specific hIPSC lines have been successfully differentiated to hepatic cells and the functionality of these human hepatocytes has been fully characterized in the GUNN rat, model of Crigler-Najjar diease. The

patient-specific iPSC line have also been corrected and mutated function restored. Finally conditions to decrease bilirubin level in Gunn rat have been defined with newborn hepatocytes, a differentiation stage corresponding to what is achieved with ES/iPSC differentiation protocol, as well as conditions to improve hepatocyte engraftment and an immunodeficient GUNN rat model is in the process of being created.

Altogether, the findings of *InnovaLiv* have already been published in journals of high impact (eight articles published, one in press), and at least eight manuscripts are currently being prepared or will be written in the next future, thereby furthering the general knowledge of hESC-derived cell therapy and iPSCs-based personalized medicine and drug screening.

Collectively, the results of *InnovaLiv* provide a means for developing hepatocyte-based therapy as a standardized procedure of regenerative medicine which can be used to generate cell products for transplanting stem-cell based products into patients with life-threatening metabolic diseases. The methods and tools developed by *InnovaLiv* will now be available to the academic community to facilitate research on hESC and hiPSC**.** Moreover the models of the 3 metabolic diseases induced pluripotent stem cell models which opens up all the field of in vitro liver disease modeling to understand the basis of molecular gene regulation but also in the case of Familial Hypercholesterolemia the role of LDLR receptor in different virus entry.

At an economic level, various products generated by *Innovaliv* including medium and GMP-grade reagents for large-scale culture of hESC will be commercialized by the 2 SMEs with the aim of advancing cell-based therapies through the availability of these much needed tools for clinical applications.

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3	Fundación de la Comunidad Valenciana Centro de Investigación Príncipe Felipe	Deborah Burks	CIPF	Spain
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PROJECT LOGO AND PUBLIC WEBSITE

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