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Obtaining hepatocyte-like cells from dental pulp pluripotent-like stem cells.

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Obtaining hepatocyte-like cells from dental pulp pluripotent-like stem cells

PhD Thesis

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Life is the art of drawing sufficient conclusions from insufficient premises

Samuel Butler

(United Kingdom, 1835-1902)

La vida es el arte de sacar conclusiones suficientes a partir de premisas insuficientes

Samuel Butler

(Reino Unido, 1835-1902)

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INDEX

ABBREVIATIONS

AAT: Alfa 1-antitrypsin
ADMSC: Adipose tissue mesenchymal stromal cells
AFP: Alfa fetoprotein
ALF: Acute liver failure
ALP: Alkaline phosphatase
ALT: Alanine aminotransferase
ASC: Adult stem cells

AST: Aspartate aminotransferase
BAL: Bioartificial liver support
BM: Bone marrow
BMP: Bone morphogenic protein
CGH: Chromosome genomic hibridization
CK: Cytokeratin
CXCR4: C-X-C chemokine receptor type 4
DEX: Dexamethasone
DFPC: Dental follicle progenitor cells
DNMT3B: DNA (Cytosine-5-)-Methyltransferase 3 Beta
DPMSC: Dental pulp mesenchymal stem cell
DPPSC: Dental pulp pluripotent stem cells
DPSC: Dental pulp stem cells
EB: Embryoid bodies
ECM: Extracellular matrix
EGF: Epidermal growth factor
EPCAM:Epithelial cell adhesion molecule

ESC: Embryonic stem cells
ESDL: End-stage liver disease
FACS: Fluorescence activated cell sorter
FBS: Fetal bovine serum
FGF: Fibroblast growth factor

FOX: Forkhead box
GGT: Gammaglutamyl transferase
GMP: Good manufacturer practices
GSC: Goosecoid homeobox
HB: Hepatoblasts
HCC: Hepatocellular carcinoma

HHEX: Hematopoietically expressed homeobox

HLC: Hepatocyte like-cells
HNF: Hepatocyte nuclear factor
HNF4: Hepatocyte nuclear factor 4

HPC: Hepatic progenitor cells
HT: Hepatocyte transplantation
ICAM: Intercellular adhesion molecule
iPSC: Induced pluripotent stem cells
LIF: Leukemia inhibitor factor
LPC: Lateral population cells
MAPC: Multipotent adult progenitor cell
MIAMI: Marrow-isolated adult multilineage inducible
MPC: Mesodermal progenitor cell
MSC: Mesenchymal stromal cells
NAFDL: Non-alcoholic fatty liver disease
NCAM: Neural Cell Adhesion Molecule
OC-2: One cut homeobox-2
OCT4: Octamer-Binding Protein
OLT: Orthotopic liver transplantation
OSM: Oncostatin M
PCR: Polymerase Chain Reaction
PDGF: Platelet derived growth factor

PDLSC: Periodontal ligament stem cells
PKU: Phenylketonuria
PROX: Prospero homeobox
REX1: ZFP42 Zinc Finger Protein
SCAP: Stem cells from apical papilla
SEM: Scanning electron microscopy
SHED: Stem cells from human exfoliated deciduous teeth
SOX: Sex determining region Y-box
SSEA: Stage specific embryonic antigen
STM: Septum transversum mesenchyme
TBX3: T-Box 3
TDGF1: Teratocarcinoma-Derived Growth Factor 1
TEM: Transmission Electron Microscopy
TGF: Transforming growth factor
TNF: Tumor necrosis factor
TO: Tryptophan 2-3-dioxygenase
TRA: Tumor-rejection antigen
UCMSC: Umbilical cord mesenchymal stromal cells
UDPGT: Uridine diphosphate glucuronosyltransferase
USA: United States of America
VSEL: Very small embryonic-like
WNT: Wingless-Type MMTV Integration Site Family

INTRODUCTION

Justification

The liver is the largest internal organ providing essential metabolic, exocrine and endocrine functions including the production of plasma proteins, protease inhibitors, blood coagulation factors, regulation of glucose, amino acids and fatty acids metabolism and, moreover, plays a critical role in detoxification.
Since the liver is such an important regulator of homeostasis, liver diseases as hepatic fibrosis, cirrhosis, hepatitis and hepatocellular carcinoma result in high rates of morbidity and mortality. End stage liver disease (ESLD) is an irreversible condition that leads to the eventual failure of the liver and its current optimal treatment is orthotopic liver transplantation (OLT). One of the greatest hurdles that liver transplantation faces today is the lack of available organs.

Hepatocyte transplantation is a promising alternative to OLT for several applications, but the problems it faces are similar to OLT due to the requirements on the number of hepatocytes needed for an adult liver.

Therefore, a lot of efforts are being made by the scientific community to obtain hepatocyte-like cells from alternative sources other than the human adult liver. One of the main focuses of regenerative medicine in the last years has been to obtain a population of hepatocyte-like cells from stem cells. At first, the strategies were focused mainly in embryonic stem cells (ESC) but some years later other possible sources have risen as potential candidates.

Dental pulp pluripotent stem cells (DPPSC) are a very recently discovered source of adult stem cells (ASC) that have been shown unique differentiation capabilities similar to embryonic stem cells. They reunite characteristics typical from adult stem cells but with a larger plasticity thanks to their embryonic-like abilities.

In this work we propose that DPPSC are a good candidate to generate hepatocyte-like cells as they have pluripotent-like characteristics without the need of genetic manipulation, also without any ethical inconvenience and, most importantly, they can be expanded to an unlimited number of cells allowing autologous transplantation.

The liver

The liver is the largest internal organ providing essential metabolic, exocrine and endocrine functions. Hepatocytes are parenchymal cells and comprise approximately 70% of the adult liver mass and along with cholangyocytes, the epithelial cells of the bile duct, are derived from the embryonic endoderm (1). The other cells from the liver (stellate cells, kuppfer cells, liver sinusoid) have a mesodermal origin and carry out functions complementary to those of the hepatocytes. Stellate cells serve as reservoirs of vitamin A and they are the primary extracellular matrix-producing cells in the liver (2). The kupffer cells are specialized macrophages located in the liver (3) and the liver sinusoid are endothelial cells characterized by their open pores and the lack of basal lamina (4).

Hepatocytes are polarized cells and are arranged as cords that are one cell thick. The basolateral surfaces of the hepatocyte face fenestrated sinusoidal endothelial cells which facilitate the transfer of endocrine secretions from the hepatocytes into the blood stream. The histologically best-defined structural unit of the liver is the lobule. It has a hexagonal shape and consists of hepatocyte plates which radiate from a central vein in the center of the hexagon (Figure 1). Adjacent hepatocytes are joined by tight junctions that delimit the bile canaliculi. Each of six corners is demarcated by the presence of a portal triad of vessels consisting of a portal vein, bile duct and hepatic artery. The human liver contains about one million lobules. The liver is not particularly rich in extracellular matrix (ECM). However, ECM plays an important role in maintaining the differentiated phenotype of hepatocytes. Significant alterations in the ECM are found in liver cirrhosis and fibrosis. (5)

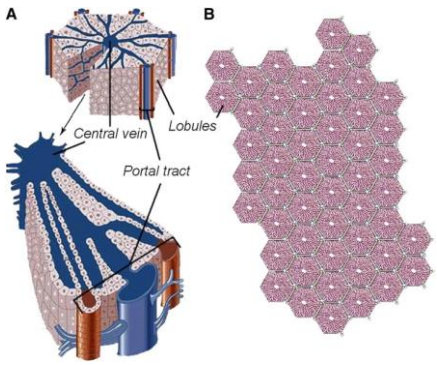


Figure 1. Schematical representation of the hepatic lobules. Extracted from Gulec et al. (6)

To have an idea of the importance of hepatocytes in maintaining body homeostasis, it is important to know that they produce the majority of circulating plasma proteins including transporters (albumin, ceruloplasmin, transferrin, lipoproteins), protease inhibitors (α1-

antitrypsin, antithrombin, α2-macroglobulin), blood coagulation factors (fibrinogen, prothrombin, factors V, VII, IX, X, etc) and modulators of immune complexes and inflammation (complement C3, C-reactive protein). They also control the homeostasis of glucose/glycogen and fatty acids such as triglycerides, cholesterol, bile acids, and vitamins A and D. Hepatocytes metabolize amino acids, metals (cupper and iron) and endogenous compounds such as heme and bilirubin. In addition, they play a critical role in detoxifying xenobiotics and drugs (7).

Liver disease

Since the liver is such an important regulator of homeostasis, liver diseases result in high rates of morbidity and mortality. Liver disease is, actually, the fourth leading cause of death among middle-aged adults in the United States. Some risk factors for liver disease include obesity/overweight (8), alcoholism (9), exposure to industrial toxins and long-term use of certain medications (10). Age and gender also have an influence on liver disease but the factors vary depending on the particular type of disease (11).

One example of liver disease is cirrhosis that accounts for 1.8% of all deaths in Europe (170,000 per year) (12). Alcohol is the strongest risk factor but infections by the hepatitis B and C viruses have a big influence on it. In turn, liver cirrhosis can lead to hepatocellular carcinoma.

Liver cancer is responsible for 46,801 deaths per annum in Europe becoming the fifth most common malignancy (13). Hepatocellular carcinoma (HCC) is the most common liver cancer (80-90%). In 2008 liver cancer incidence in Europe was 10.6 and 3.6 per 100,000 inhabitants for men and women, respectively. In Spain it was 12 and 3.5 per 100,000 respectively (14). Infection by the hepatitis virus is a risk factor frequently associated with liver cancer; carriers of hepatitis virus B have 100 times more risk of developing liver cancer.

Incidence rate of hepatitis A in Europe is between 0.55 and 1.5 cases per 100,000 habitants. That of hepatitis B is between 0.2 and 11.2 (1.5 in Spain) per 100,000 habitants and causes death of 2.7 persons per 100.000 habitants in Spain every year. In the case of hepatitis C, a significant number of people acquired it in the 1970s and 1980s before the virus was identified. Since then, the transmission has vastly decreased. The current annual average incidence rate is estimated at 6.19 per 100,000 inhabitants. In Spain, the mortality rate due to HCV infection is 11.25 per 100,000 inhabitants (12).

Non-alcoholic fatty liver disease (NAFLD) is defined as the accumulation of liver fat exceeding 5% of hepatocytes in the absence of significant alcohol intake viral infection, or any other specific etiology of liver disease. Obesity is the main risk factor, therefore NAFLD can be considered to be a major potential threat to public health in Europe. Diabetes is another risk factor related to it. In two recent studies the prevalence of NAFLD was stablished around 26% (15, 16).

All of these hepatic disorders can lead to ESLD and to an eventual failure of the liver. The incidence of ESLD is increasing worldwide(12), and the current optimal treatment for ESLD is OLT which is nowadays a routine procedure with five-year survival of more than 70% (17). More than 5,500 liver transplants are currently performed in Europe every year (Figure 2). In 2012 there were approximately 17,000 people on the waitlist for liver transplantation in the USA alone. However, one of the greatest obstacles that liver transplantation faces today is the growing discrepancy between the demand of organs and the availability of donors. In fact, 15% listed patients die while awaiting a graft (18).

	Overall	LRLT
AUSTRIA	2193	38
BELARUS	34	0
BELGIUM	4316	341
BULGARIA	30	15
CZECH REPUBLIC	1089	1
DENMARK	734	8
ESTONIA	12	0
FINLAND	801	0
FRANCE	16,366	409
GERMANY	14,116	715
GREAT BRITAIN	13,684	138
HUNGARY	425	3
IRELAND	653	0
ITALY	11,697	212
MONACO	11	0
NORWAY	734	1
POLAND	1462	135
PORTUGAL	1645	26
ROMANIA	206	47
SLOVENIA	140	0
SPAIN	15,714	189
SWEDEN	2163	54
SWITZERLAND	1475	59
THE NETHERLANDS	1972	14
TURKEY	1946	1217
EX-YUGOSLAVIA	16	1
TOTAL	93,634	3622

Figure 2. Number of liver transplantations in European countries, May 1968 to December 2009; ELTR(19).

Hepatocyte transplantation as an alternative to OLT

Taking into account the shortage of livers to be transplanted, hepatocyte transplantation (HT) is a promising alternative to OLT for many clinical applications. The procedure is less invasive than OLT and can be performed repeatedly. It can be especially useful in liver based inborn errors of metabolism where the aim is to replace a single deficient enzyme or its product.

The hepatocytes used in HT are isolated from liver tissues rejected/unused for transplantation, including livers from non-beating heart donors. The protocol for the isolation of human hepatocytes is well-established and consists on collagenase digestion of perfused liver tissue at 37ºC. Once the liver tissue is digested and cells are released, the hepatocytes are purified by centrifugation and assessed for cell viability and yield (20). Cells are either transplanted immediately after isolation or cryopreserved which are needed for emergency treatment and for repeated use of cells from the same donor. However, cryopreservation is detrimental on the viability and metabolic functions of hepatocytes. Although optimization of protocols has been developed in recent years, there is still a need for further studies to increase the efficiency of cryopreservation of hepatocytes. In addition, differences in cell preparation and clinical protocols make it difficult to compare the results of human hepatocyte transplantation between different centers (21).

Problems related to the low engraftment efficiency and cell function declining after a few months are the biggest obstacles in these therapies. Many transplanted cells do not survive long after transplantation mainly because they do not integrate into the liver structure due to several reasons. In one hand, the immune system rapidly clears a high percentage of cells mainly through Kupffer cells. In the other hand, the activation of the coagulation cascade and complement also difficult the survival of the transplanted hepatocytes albeit patients receiving immunosupressors. Moreover, a high number of cells usually is ectopically distributed which lowers the efficiency and can derive into emboli formation.

The monitoring of cell engraftment is, in addition, pretty difficult to carry out. It can be obtained from levels of metabolic products when treating inborn metabolic errors, but this may not provide reliable information about the location or number of functioning cells (21). All these issues can lead to only transient effects of the transplanted hepatocytes.

Clinical approaches using hepatocyte transplantation

Worldwide, there are reports of more than 30 patients who have been treated with HT for inherited metabolic diseases, being children with urea cycle defects the main cause. It is generally accepted that the cell requirement for transplantation is lower for some of these disorders in which the goal is to replace a single deficient enzyme compared with compensating for acute liver failure. The number of cells transplanted usually represents approximately 5% of theoretical liver mass and either fresh or cryopreserved hepatocytes can be used. The safety of the procedure has been well stablished and the clinical results are encouraging with clear improvement in disease phenotype (21).

Some of the clinical trials based on hepatocyte transplantation in order to treat inherited metabolic diseases are summarized in Table I.

Reference	Disease	Number of patients	Outcome
Lysy *et al.* (22)	Crigler-Najjar syndrome	8	Decrease in serum bilirubin, uridine diphosphate glucuronosyltransferase (UDPGT) enzymes
Jorns *et al.* (23)	Glycogen storage disease type I	2	Blood sugar control improved and lactate decreased
Dhawan *et al.* (24)	Factor VII deficiency	2	70 % decrease in requirement of recombinant FVII
Hughes *et al*. (25)	Familial hypercholesterolemia	5*	Prolonged reductions in LDL cholesterol

**Hepatocytes were transduced ex vivo with a retroviral vector carrying the human LDL receptor gene*

Table I. Summary of some clinical trials using hepatocyte transplantation to treat inherited metabolic diseases.

For some metabolic disorders the risks associated with OLT are not justified, and hepatocyte transplantation could be a less invasive option to improve the long-term outcome for these patients. In phenylketonuria (PKU), for instance, the implementation of screening of newborns has enabled early initiation of lifelong dietary treatment based on a low phenylalanine diet. However, even when treatment is started early, the neurodevelopment of patients with PKU has been found to be worse than in the general population. In addition, compliance with a low phenylalanine diet decreases with increasing age and this worsens the neurological prognosis Therefore, PKU is one disease that could potentially benefit from hepatocyte transplantation (24).

Aside from metabolic diseases, other applications for hepatocyte transplantation have been tried over the last years. More than 40 patients have been treated in six centers from the United States, most of them patients with acute liver failure (ALF). Hepatocyte transplantation in patients with ALF aims to restore liver function for a period of bridging to OLT or until the native liver regenerates. To provide sufficient function the number of cells required for the treatment of acute liver failure needs to be higher than for metabolic disorders, and normally requires repeated infusions. A major advantage of hepatocyte transplantation is the immediate availability of cryopreserved cells. Patients treated in this situation had conflicting results: although they had a reduction in ammonia and bilirubin levels, HT did not significantly affect the clinical outcome of these patients (26).

Other clinical approaches for hepatocyte transplantation include their use for chronic liver disorders. Fisher and Strom describe the outcomes of 20 patients with chronic liver disorders treated with hepatocyte transplantation. Outcomes were variable, probably due to the presence of fibrosis, which makes it difficult for cells to cross the sinusoidal barrier and engraft efficiently (27).

Other applications of hepatocyte-like cells

Bioartificial liver (BAL) support

Besides direct transplantation, the generation of hepatocytes or hepatic-like cells can be very useful in extracorporeal liver devices that have been explored as a treatment to sustain patients until successful liver regeneration or until a donor organ becomes available. BAL support systems can be useful for the treatment of acute liver disease providing detoxification while also assuming synthetic roles for the failing liver. More than 30 different cell-based support devices have been reported since 1987 and 14 have been tested in clinical trials (28). However, various factors including the high cost, complexity, and difficulty associated with both obtaining and maintaining hepatocytes in a differentiated state have prevented the successful application in clinical practice. Many cell types have been studied as the biological component of extracorporeal BAL support systems. An appropriate cell source should combine the following characteristics: nearly full functionality of mature human hepatocytes, unlimited life-span and proliferative capacity in vitro and no potential risk of metastatic tumor formation, zoonotic transmission, or immunogenicity (29). Primary human hepatocytes, primary pig hepatocytes or human liver tumor-derived cell lines are the main candidates, but none has been shown to be ideal.

In Table II there is a summary of the clinical trials up to date involving BAL support. As an example, 171 patients were enrolled in a phase 3 clinical trial using porcine hepatocytes in a hollow-fiber bioreactor. The study demonstrated favorable safety and patients treated with a subsequent liver transplantation had improvements in survival compared to patients that only undergone the liver transplantation without the BAL treatment. In another case, they used the C3A hepatoblastoma cell line in a multiple hollow-fiber device (30). In this trial, transplant-free survival at 28 days was 84.1% for treated patients and 50.2% for the control group. At 84 days, transplant-free survival was 67.9% for treated patients and 43.9% for the untreated group (28).

Disease modeling

Disease-specific hepatocyte-like cells obtained upon differentiation can provide a relevant model system because their properties closely resemble those found in the patient's own system, without the need for genetic manipulation. They could generate more accurate predictions of human physiological responses than animal models. For instance, stem cell-derived hepatocytes would be useful as *in vitro* and *in vivo* models for testing hepatitis B and C virus. These hepatotropic viruses affect primarily human hepatocytes and the lack of such systems has limited the understanding of pathogenesis of these diseases, development of drugs, and testing for resistance.

Drug discovery

Human hepatocytes have a critical part to play in the drug discovery process. It is estimated that up to 50% of drug withdrawals from the market is related to hepatotoxicity (31). There is a need of a high-throughput reliable system capable of screening for hepatotoxicity and also for understanding pharmacokinetics of potential new drugs. The key requirements for such an application are easy availability of cells, consistency in metabolic capacity, and a comprehensive profile of drug metabolizing mechanisms. The pharmaceutical industry currently uses tumor-derived cells such as HepaRG (32) and primary hepatocytes to screen compounds. While these models are useful, they do not always extrapolate to human biology and exhibit poor lifespan and variable metabolic activity. Stem cell-derived hepatocytes would be extremely useful for this need. Issues of safety and tumorigenesis are less critical in this setting. Human hepatocyte-like cells (HLC) derived directly from patients show great promise for research and clinical applications as they would allow investigation into the effects of single nucleotide polymorphisms on drug metabolism and toxicity (33).

	Year	Type of trial	Number of patients	Survival advantage	Other efficacy	Adverse events
ELAD (Before modified)	1994	Phase I trial	11	NA	Improvement of neurologic status	Transient hypotension was observed in 1 patient
	1996	RCT	24	No	Not observed	Two patients were withdrawn from the study due to hypersensitivity reaction or coagulation disorder
ELAD (Modified)	2002	Phase I trial	5	NA	Stabilization of hemodynamic and respiratory function	Not observed
HepatAssist	1995	Phase I trial	10	NA	Improvement of neurologic status, decrease of ammonia	Not observed
	1999	Phase I trial	39	NA	Improvement of neurologic status, decrease of bilirubin, ammonia and transaminases	Treatable hypotension was observed in 8 patients
	2002	Phase I trial	10	NA	Improvement of neurologic status, decrease of bilirubin and transaminases levels	Six patients had transient episodes of hemodynamic instability, and five had bleeding complications
	2004	RCT	171	Only in subgroups	Decrease of bilirubin level	Data not shown
BLSS	2001	Phase I trial	4	NA	Decrease of bilirubin and ammonia levels	Treatable hypoglycemia and transient hypotension at start of perfusion
AMC-BAL	2002	Phase I trial	7	NA	Improvement of neurologic status, stabilization of hemodynamics, decrease of bilirubin and ammonia	Transient hypotension was observed in 2 patients
MELS	2002	Phase I trial	8	NA	Improvement of neurologic and coagulation status	Not observed
	2003	Phase I trial	8	NA	Decrease of bilirubin and ammonia levels	Not observed
RFB	2002	Phase I trial	7	NA	Improvement of neurologic status, decrease of ammonia and transaminases levels	Not observed
HBAL	2003	Phase I trial	12	NA	Improvement of coagulation status, decrease of ammonia and bilirubin	Data not shown
TECA-HALSS	2001	Phase I trial	6	NA	Improvement of neurologic and coagulation status, decrease of ammonia, bilirubin and transaminases levels	Not observed

Table II. Clinical trials up to date involving BAL support. Modified from Zhao et al. (29).

Alternatives to adult hepatocytes from hepatic tissues

At present the hepatocyte transplantation is limited by the availability of human hepatocytes from unused donor liver. Therefore, the scientific community has put a lot of effort into obtaining hepatocyte-like cells from sources other than the human adult liver.

The liver is known as an organ with high inherent regenerative capacity, so it should be an adequate source of stem cells to potentially treat many hepatic disorders. However, unlike other regenerating tissues (the skin, the epithelial lining of the gut, the bone marrow), in healthy conditions the liver regeneration is not dependent on stem cells or progenitor cells. It is normally carried out by proliferation of mature hepatocytes allowing full recovery of liver mass. The required proliferation of all the other mature cell types besides hepatocytes in the liver including the biliary epithelial cells, sinusoidal endothelial cells, Kupffer cells, and stellate cells remains as a much less understood process (34).

However, this regenerative capacity is overwhelmed during massive or chronic injury of livers because of the continuous loss of cells and fibrotic environment. In such cases, liver progenitor cells are activated in a process known as ductural reaction, associated with expansion of small progenitor cells in the periportal region. These progenitor cells are called “oval cells”, “hepatobiliary cells” or “hepatic progenitor cells” (HPC). They are bipotential cells and can differentiate into both cholangiocytes and hepatocytes depending upon the epithelial cell-type which incurs the most damage (Figure 3). They have large oval shaped nucleus and scanty cytoplasm and can be found in the ductal plates of fetal and neonatal livers, and canals of Hering in pediatric and adult livers. Their phenotypic profile includes EpCAM, NCAM, CD133, CXCR4, SOX9, SOX17, FOXA2, CK 8/18/19. They do not express AFP, ICAM-1 or markers for hematopoietic, endothelial or mesenchymal cells. They can be isolated by dual immunoselection for EpCAM+/NCAM+ cells from livers of all donor ages. In culture, HPC form colonies capable of self-replication and of differentiation to mature cells in culture and *in vivo* (35). However, the clinical application of this cell source is limited due to the difficulty in obtaining large numbers cells.

Although the exact mechanisms controlling progenitor activation in chronic liver injury remains unclear, collective data suggest that progenitor-mediated regeneration of the adult liver may follow a pattern similar to fetal development. The tissue microenvironment plays an essential role in orchestrating oval cell-mediated liver regeneration (36).

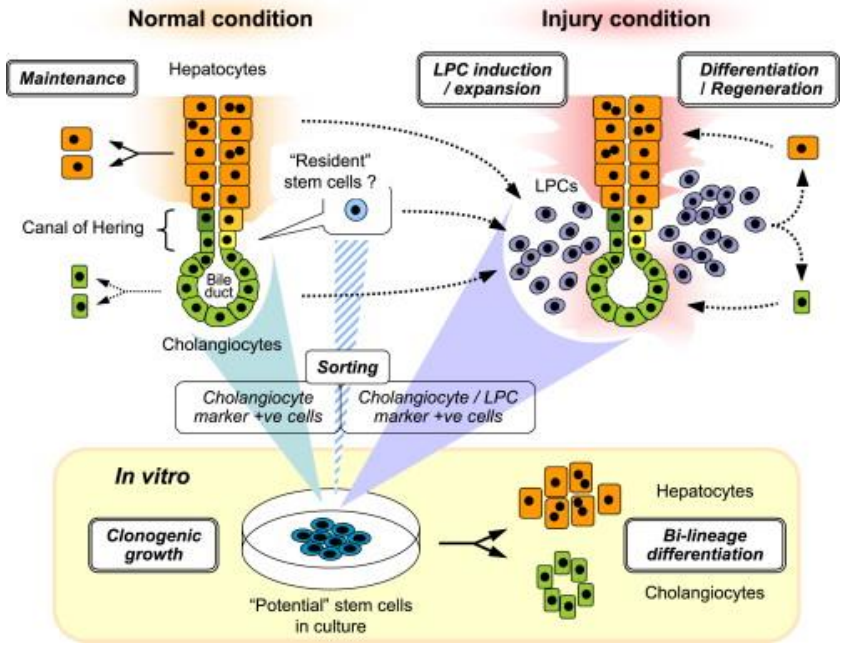


Figure 3. Schematic representation of liver regeneration in normal and injury condition. Extracted from Miyajima et al. (37)

Similar to HPC, hepatoblasts (HB) from fetal liver could also represent a potential source of hepatocytes and biliary cells. There are still limitations to widespread use of these cells, such as low cell number *in vivo*, no specific biomarkers for purification and poor expansion *in vitro*. Percentage of human HB in postnatal liver is very low, they represent around 0.01% of the parenchymal cells (38).

Hepatocytes from other mammals (primarily porcine) are also under study but they suffer as well from rapid decrease in functional activities when cultured *in vitro*. In addition, compared to human hepatocytes there are some differences in drug metabolism and other hepatic functions that together with immunogenic concerns make them hard to use (39).

Liver cell lines have been employed as they demonstrate long lifespan and are easy to maintain but they usually show lower drug-metabolizing activity than their adult counterparts and do not accurately predict human drug toxicity and therefore do not constitute a real alternative to the gold standard primary hepatocytes (40). HepG2 is a liver cell line derived

from fetal tissue which exhibits poor metabolic function and secretes a variety of soluble serum proteins. C3A, a subclone of HepG2 has been widely studied and are currently the only cell line used in a clinical trial for a bioartificial liver device. However, there are some concerns as they express lower levels of ammonia detoxification, urea cycle, activity P450 activity and amino acid metabolism (41). HepaRG cells have also studied and they have demonstrated a number of liver-specific functions including the expression of several cytochromes (42). Because of the potential for tumor transmission, the clinical use of immortalized cell lines has been limited to extracorporeal devices with membrane for blocking the spread of cells to the patients (43).

Liver development

The liver as a complex organ includes different cells with diverse embryological origin. Hepatocytes and cholangyocytes are derived from the embryonic endoderm, while the stromal cells, stellate cells, kuppfer cells and blood vessels are of mesodermal origin. As hepatocytes represent the most important cell in the liver, their embryological development is the most interesting to examine. Studies in animal models such as mouse, chicken, zebrafish and *Xenopus* show that hepatogenesis is evolutionary conserved and occurs through a progressive series of reciprocal tissue interactions between the embryonic endoderm and nearby mesoderm (44).

Definitive endoderm emerges as a sheet of cells from the anterior end of the primitive strike during gastrulation. Nodal is a member of the TGFβ superfamily and it is essential for the specification of endoderm during gastrulation. Nodal initiates both endoderm and mesoderm formation in a concentration-dependent manner, as follows: high levels of Nodal specify definitive endoderm and low levels mesoderm (45).

Nodal signaling stimulates the expression of a core group of endoderm transcription factors including Sox17 and the fork head domain proteins Foxa1-3 which in turn regulate a cascade of genes committing cells to the endoderm lineage (46).

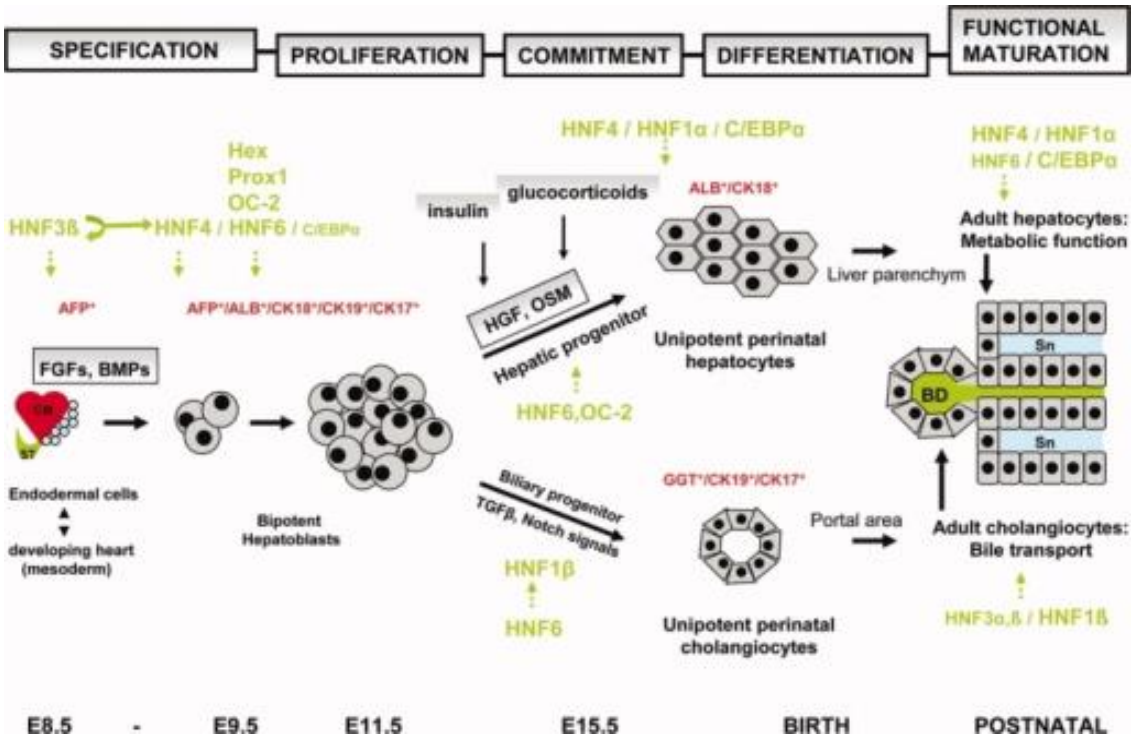


Figure 4. Schematic representation of liver development. Extracted from Snykers et al. (47)

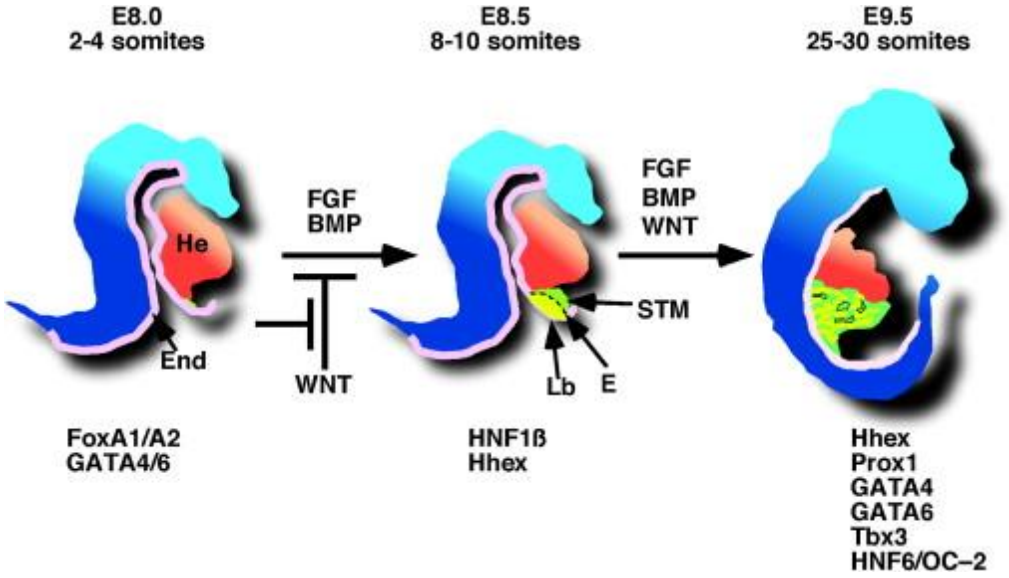


Figure 5. Scheme representing the development of the liver. Extracted from Si-Tayeb et al. (7)

Throughout gastrulation morphogenetic movements turn the endoderm into an epithelial gut tube surrounded by mesoderm. The gut tube is further patterned along the anterior-posterior axis into foregut, midgut and hindgut that can be identified by the expression of specific transcription factors such as Hhex in the foregut, Pdx1 in the midgut and Cdx in the hindgut. The foregut contains the common precursors of the liver, gall bladder, pancreas and lungs. FGF4 and Wnts secreted from the posterior mesoderm repress foregut fate and promote

hindgut development, while to stablish the foregut identity Wnt and FGF4 signaling must be inhibited in the anterior endoderm (48) (Figure 4).

Several Gata family members are acting downstream of Nodal although the proteins leading to Gata activation are different in several model organisms. Foxa family and Gata factors are key players of the endodermal network of transcription factors in all triploblasts. Their inactivation perturbs but does not abolish endoderm development. Foxa2 is expressed at the onset of gastrulation and is required for fore- and midgut formation. Sox17 is another key component downstream of Nodal. In mouse it is expressed in visceral endoderm and knockout mice do not form definitive endoderm (44).

FGF signals from the developing heart and BMPs from the septum transversum mesenchyme (STM) induce hepatic fate in the ventral foregut endoderm. If the cardiac mesoderm is removed or if either FGF or BMP signaling is blocked, liver induction does not occur (1). The newly specified hepatic cells are referred to as hepatoblasts at this stage and they can give rise to both hepatocytes and cholangyocytes (Figure 5).

Shortly after hepatic specification the epithelium begins to express liver genes (albumin, AFP, Hnf4) and the cells transition from a simple cuboidal shape to a columnar epithelium forming the liver diverticulum. Wnt signaling, initially repressed, becomes necessary to promote liver bud emergence. The hepatoblasts then delaminate and migrate into the STM to form the nascent liver bud. Liver bud formation is controlled by a network of transcription factors including Hhex, Gata-6, HNF6, OC2, Tbx3 and Prox-1. Once hepatoblasts bud into the local mesenchyme they continue to proliferate under the influence of cytokines such as FGF, EGF, HGF, TGf-β, TNF-α and IL-6 secreted by mesenchymal cells (49). Prior to vascularization of the liver bud, endothelial precursor cells lay between the hepatic epithelium and the STM. Endothelial cells provide paracrine factors promoting hepatoblasts migration and/or proliferation. After that, the liver bud undergoes great growth and becomes the major site of fetal haematopoiesis (50).

Initially hepatoblasts express genes associated with adult hepatocytes (Hnf4α, Albumin) and cholangyocytes (Ck19) as well as fetal liver genes (AFP). Hepatoblasts in the liver parenchyma that are not in contact with portal veins gradually differentiate into mature hepatocytes. They acquire their epithelial morphology arranged in hepatic chords with bile canaliculi on the apical surfaces. In contrast, hepatoblasts in contact with the portal vein form a monolayer and then a bi-layer of biliary precursor that increase expression of CK19 and down-regulate hepatic genes. Hematopoietic cells in the liver secrete Oncostatin M (OSM) which in combination with HGF

and Wnt promotes hepatic differentiation. TNFα balances the differentiation by inhibiting maturation and maintaining the proliferative capacity of fetal hepatocytes, allowing the liver to grow enough before differentiating. HNF4a is involved as well in the expression of many mature hepatic enzymes as it binds to nearly half of the genes expressed in the mouse liver. Conditional deletion of HNF4a in fetal hepatocytes results in the faint expression of many mature hepatic enzymes and the impairment of normal liver morphology (51).

Stem cells

Stem cells are unspecialized cells that can self-renew indefinitely and that can also differentiate into more mature cells with specialized functions. In humans, stem cells have been identified in the inner cell mass of the early embryo; in some tissues of the fetus, the umbilical cord and placenta; and in several adult organs. Intense research on stem cells during the last decades has provided important information on developmental, morphological, and physiological processes that govern tissue and organ formation, maintenance, regeneration, and repair after injuries. More recently, significant advancements in our understanding of stem cell biology have provoked great interest and hold high therapeutic promise based on the possibility of stimulating their ex vivo and in vivo expansion and differentiation into functional progeny that could regenerate injured tissues/organs in humans (52).

ESC are derived from an early-stage embryo. Fertilization of an ovum by a sperm results in a zygote, the earliest embryonic stage. The zygote begins to divide about 30 hours after fertilization and by the third-to-fourth day, the embryo is a compact ball of 12 or more cells known as the morula. Five-to-six days after fertilization, and after several more cycles of cell division, the morula cells begin to specialize, forming a hollow sphere of cells, called a blastocyst. The outer layer of the blastocyst is called the trophoblast, and the cluster of cells inside the sphere is called the inner cell mass. At this stage, there are about 70 trophoblast cells and about 30 cells in the inner cell mass. The cells of the inner cell mass are the ESC, pluripotent stem cells that give rise to all cell types of the major tissue layers (ectoderm, mesoderm, and endoderm) of the embryo (53).

Human embryonic stem cell (hESC) lines express many markers that are common to pluripotent and undifferentiated cells, such as CD9, CD24, OCT4, NANOG, ALP, LIN28, Rex-1, Cripto/TDGF1, DNMT3B, SOX2, EBAF, and Thy-1, as well as stage-specific embryonic antigen-3 and -4 (SSEA-3 and -4) and tumor-rejection antigen-1-60 and -1-81 (TRA-1-60 and -1-81) (54).

In 2006, Takahashi *et al.* (55) showed that the introduction of four specific genes encoding transcription factors could convert adult cells into pluripotent stem cells kwon as induced pluripotent stem cells (iPSC). They have the ability to propagate indefinitely, to give rise to every cell in the adult body and they are similar to ESC in morphology, proliferation and teratoma formation.

Adult stem cells are undifferentiated cells that reside in most of adult tissues/organs, including bone marrow, heart, brain, lungs, liver, skin, eyes, etc. (38, 56-60) They can renew themselves in the body, making identical copies of themselves for the lifetime of the organism, or become specialized to yield the cell types of the tissue of origin (61).

One group of adult stem cells, mesenchymal stromal cells (MSC), has generated great interest in the fields of regenerative medicine and immunotherapy due to their unique biologic properties. MSCs were first discovered in 1968 by Friedenstein and colleagues (62) as adherent fibroblast-like cells in the bone marrow (BM) capable of differentiating into bone. It was subsequently shown that MSCs could be isolated from various tissues such as BM, adipose tissue (63), and umbilical cord blood (64). These cells can be expanded *in vitro*, which allows them to rapidly reach the desired cell counts for use *in vivo*. Using somewhat different strategies, several laboratories have identified, isolated, and cultured MSCs with specific properties (65).

Stem cells from the dental pulp

Dental pulp is a soft connective tissue within the dental crown thought to be derived from migratory neural crest cells during development. It has been shown to harbor various populations of multipotent stem/progenitor cells. Since their very first isolation in 2000 by Gronthos *et al.* (66), several types of adult stem cells have been isolated from teeth, including dental pulp stem cells (DPSC), stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSC), dental follicle precursor cells (DFPCs), and stem cells from apical papilla (SCAPs)(66-70).

These post-natal populations have MSC-like qualities, namely the capacity for self-renewal, the potential to differentiate into multiple lineages including osteoblasts and chondroblasts, and a potential for in vitro differentiation into cell types from various embryonic layers, including adipose, bone, endothelial and neural-like tissue. They are often compared to BMMSC and share many similarities with them, but they differ in that dental stem cells seem to be

committed to an odontogenic fate, more so than to an osteogenic one. Since they have a neural crest origin, they have stronger neurogenesis capabilities but weaker adipogenesis and chondrogenesis (71).

DPSCs are successfully isolated by enzymatic digestion of pulp tissue after separating the crown from the roots. These cells are morphologically similar to fibroblasts, very proliferative and clonogenic. Dental pulp stem cells are multipotent cells that proliferate extensively, can be safely cryopreserved, possess immunosuppressive properties and express markers such as CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD146, and STRO-1, but do not express CD14, CD24, CD34, CD45, CD19 and HLA-DR. They have the ability to differentiate into odontoblast-like cells, osteoblasts, adipocytes, neural cells, cardiomyocytes, myocytes, and chondrocytes in vitro. DPSCs represent less than 1% of the total cell population present in the dental pulp (72).

Miura *et al.* (67) reported the potential to obtain stem cells from human deciduous teeth. They are derived from dental pulp explants or by digestion of dental pulp tissue and have immunosuppressive properties. They have a higher proliferation rate than BMMSC and DPSC and have a higher expression in genes that participate in pathways related to cell proliferation and extracellular matrix, including several cytokines such as FGF and TGFβ (73).

Dental Pulp Pluripotent-like Stem Cells

In previous studies, our group has described a new population of adult stem cells called DPPSC (74). These cells are isolated from the dental pulp from the third molars and express pluripotency markers such as OCT4, NANOG and SOX2, and also showed embryonic-like behaviour differentiating into tissues of the three embryonic layers.

DPPSC are not the first stem cell population isolated from adult tissues that presents pluripotency-like capacities. In this regard, several populations have been identified in recent years; marrow-isolated adult multilineage inducible (MIAMI) (75), very small embryonic-like (VSEL) (76), multipotent adult progenitor cells (MAPC) (77) and mesodermal progenitor cells (MPC) (78).

One of the main advantages from DPPSC compared to other populations is that the third molars are a very accessible source of cells, because wisdom tooth extraction is widely performed and the teeth are usually considered to be medical waste. Because the third molar is the last tooth to develop in humans, it is normally in an early stage of development and is

capable of yielding an optimum quantity of dental pulp tissue for the isolation of stem cells. Although the percentage of DPPSCs decreases with age, a population of these cells was always present, even in older patients (74).

DPPSCs are obtained from dental pulp using the same isolation protocol as DPSC and they share some characteristics. However, they differ in the expression levels of embryonic markers as well as some membrane proteins such as CD73. The culture conditions between them are also different; DPPSC need low density and a specific medium that contains growth factors such as EGF, PDGF and LIF to allow maintenance of the pluripotent state of DPPSCs. The characteristics unique to these cells are still under investigation, but the current evidence gain insights for future comparative studies of the regenerative potency of DPPSCs and stem cells from other sources. It has been demonstrated, for instance, that DPPSC have a greater capacity for generating bone-like cells in comparison with DPSC (79), and in addition, no chromosome abnormalities have been shown when DPPSC are cultured *in vitro* (74).

Hepatocytes from stem cells: differentiation protocols

Hepatocytes from embryonic stem cells

One of the main focuses of regenerative medicine in the last years has been to obtain a population of hepatocyte-like cells from stem cells. At the beginning all the strategies were based in the use of ESC as the only possible source of hepatocyte-like cells, but some years later other sources have risen as possible alternatives.

The early literature favored protocols using embryoid bodies (EB) derived from ESC which undergo gastrulation-like events. In EB, human ESC undergo spontaneous differentiation into the three germ layers, among them endodermal cells expressing AFP and albumin(80). It has been demonstrated that hESC can differentiate into hepatic-like cells through the intermediates step of EB formation. They showed that albumin-expressing cells isolated by fluorescence activated cell sorting (FACS) were capable of growing *in vitro* for a few weeks. Subsequent optimization of growth factors, ECM, and medium selection enhanced the purity of the differentiation process using EB. In addition, it was suggested that plating EB in a 3D collagen type I scaffold with the sequential addition of growth factors is more efficient than applying 2D cultures (81). However, hepatocyte differentiation through EB is no longer considered the most effective approach. Low differentiation rate, high heterogeneity and

disproportional exposure to exogenous factors limited the expansion and differentiation of the cells. Together with the lack of knowledge regarding the mechanisms of gene activation in a 3-dimensional EB promoted an increasing interest in monolayer-based protocols.

Based on the knowledge of endoderm development, strategies have more recently been devised to generate endoderm mimicking embryonic development. As *in vivo*, Nodal is necessary for endoderm induction from ESC. Due to the limited availability and cost of biologically active Nodal protein, most protocols have made use of Activin A as a surrogate for Nodal (82). Several experiments have shown that endoderm is efficiently generated with ES cells from both mouse and human species (83, 84). The low serum limits phospatidyl Inositol 3 kinase activity, a condition needed for definitive endoderm formation from ES cells. Exposure to high concentrations of Activin A favors endoderm formation while low concentration gives rise to skeletal muscle markers. At least 6 days of treatment are needed for expression of Sox17 and Hex. A shorter time can also work if other cytokines are added (44).

Most hepatocyte differentiation protocols start with Activin A alone or with Wnt3a for 3-5 day period to induce definitive endoderm commitment, as they have been the 2 combinations with greater success (85). It has been shown that HGF, BMP4, and FGF4 have no significant effect on endodermal differentiation (86). However, FGF and BMP play a significant inductive role in promoting endodermal progenitor maintenance and expansion and could subsequently be used in the second stage of directed differentiation. Combinations of BMP2, BMP4, FGF1, FGF2 and FGF4 are frequently used for hepatic induction (87, 88). The last stage often involves the use of OSM and HGF mixed along Dexamethasone to induce the maturation stage (89-91).

The differentiation of hESC into hepatocytes was first demonstrated by Rambhatla *et al*. in 2003 (92). Since then, many studies have focused on enhancing the culture conditions to obtain a more homogeneous cell population. However, success in this regard has been limited as hepatocyte-like cells obtained have more transcripts in common with fetal liver than with adult liver cells, suggesting that they may contain immature and progenitor-like cells rather than mature hepatocytes. Low level expression of cytochrome P450 transcripts and persistent expression of AFP are some of the common features of hepatocyte-like derived cells (93). Nonetheless, in recent years with a better understanding of liver development, several combinations of growth factors have come to use and have made possibly to effectively differentiate hESC to hepatocyte lineage with evidence of repopulation of immunosuppressed mouse model of liver injury shown. Some of these promising results, as well as a brief description of the cytokines used for differentiation, are summarized in Table III.

Reference	Differentiation protocol	Remarks
Agarwal *et al.* (89)	Activin A (5d); FGF4, HGF (6d); FGF4 HGF, OSM, DEX, Single Quotes (9d)	High-yielding procedure for the generation of hepatocytes from hES cells *in vitro*
Basma *et al.* (94)	EB formation (2d); Activin A, FGF2 (3d); DMSO, HGF (8d); DEX (3d)	Functional human hepatocytes from hES cells and transplantation into mice.
Duan *et al*.(87)	Activin A (2d); Activin A, NaB (3-6d); FGF4, HGF, BMP2, BMP4, DMSO (10-14 d); FGF4, HGF, OSM, DEX, DMSO, SingleQuotes (until use)	Highly efficient protocol to generate a population of functional hepatocytes from hESC.
Hay *et al.* (85)	Activin A, NaB (4d); DMSO (7d); HGF, OSM(7d)	Simple and relatively economic strategy to differentiate hESC to hepatocytes.
Ramasamy *et al.* (90)	Activin A, Nab (3d); DMSO (5d); HGF, OSM (4-5d)	3D culture enhances hESC-DE cell differentiation to HLCs and improves the resulting hepatocyte function
Roelandt *et al.* (95)	Activin A, Wnt3a (2d); Activin A(2d); BMP4 (4d); FGF1 (4d); HGF (16d)	Infection with HCV of hepatocytes derived from hESC
Touboul *et al.* (88)	Activin A, FGF2 (2d); Ly294002, Activin A, BMP4, FGF2 (3d); FGF10 (3d); FGF10, RA, SB431542 (2d); FGF4, HGF, EGF (10d).	Functional hepatocytes from human embryonic stem cells under chemically defined conditions
Woo et *al.* (91)	EB formation (6d); HGF, OSM, DEX (20d)	Human ES cell–derived hepatocytes-like cell grafts and their secretome contribute to endogenous host liver regeneration

Table III. Different protocols used to obtain hepatocytes from ESC.

The cell-based approaches are very encouraging, but further studies are required to demonstrate long-term safety of cell-based transplantation. Despite the successful animal studies, there have been no clinical trials using human ES cells to treat liver diseases in human patients because utilization of human ES cells has serious hurdles to overcome first (96). They have a long population doubling time and they need to be first scaled up in large numbers before they can be applied to patients. Large numbers of cells are required for administration for each patient and they have to be manufactured under current good manufacturing practices (GMP) and according to regulation of agencies. ESC therapies also face immunorejection problems so the transplanted cells or derived tissues may require surgical removal should there be a host versus graft reaction. The safety of the transplanted cells is another important problem to overcome. Teratoma formation has been reported when ESC or cells derived from ESC have been transplanted into immunosuppressed mice, even though

there was successful engraftment and functional improvement (94). Although previous differentiation is carried out *in vitro*, there is a need to ensure that no renegade undifferentiated hESC are transplanted together with the derived tissue. Aside of these technical issues, ethical concerns are raised with the use of hESC (97).

Hepatocytes from induced pluripotent stem cells (iPSC)

With the successful reprogramming of human somatic cells into iPSC by Takahashi *et al.* (55) and Park *et al.* (98), a new field in regenerative medicine was opened. As they shared many features with ESC but with the advantage of being obtained from an adult source, a lot of effort was put on obtaining several adult tissues from iPSC, amongst them the hepatic tissue. The knowledge previously acquired in differentiation with ESC was successfully applied in iPSC and several reports of efficient methods to obtain hepatocytes *in vitro* have been published. A summary of some of these reports is shown in Table IV.

Reference	Differentiation protocol	Remarks
Sullivan *et al.* (99)	Activin A, Wnt3a (d), Activin A (2d), DMSO (3d), HGF, OSM (6d)	Generated functional hepatocyte-like cells from human-iPSCs
Song *et al*. (100)	Activin A (3d), FGF4, BMP-2 (4d), HGF, KGF (6d), OSM, Dex (5d) then OSM, Dex, N2B27 (3d)	iPSCs had fewer expressed liver-enriched genes compared with human hepatocytes
Si-Tayeb *et al.* (101)	Activin A (5d), bFGF, BMP-4 (5d), HGF (5d), OSM (5d)	Transplanted hepatocyte-like cells into the lobe of newborn mice and demonstrated homing of donor cells
Liu *et al.* (102)	Activin A (5d), FGF4, HGF (5), Single Quotes (lonza), FGF4, HGF, OSM, Dex (10 d)	Human hepatocyte-derived iPSCs are able to differentiate into functional hepatocytes
Takata *et al.* (103)	Activin A (3d), HGF (5d), OSM (5d)	Generated hepatocyte-like cells from iPSCs using three growth factors in a short time
Asgari *et al.* (104)	Activin A (3d), FGF4, HGF (8d), Single Quotes(Lonza), Dex, OSM (2d)	Hepatocyte-like cells generated from hiPSCs that improve the fibrotic mouse model after their transplantation

Table IV. Different protocols used to obtain hepatocytes from hiPSC. Modified from Subba et al. (105)

In recent times, due to improvements on gene transfection, the success on the generation of iPSC has caused a focus on directing liver cell fate through gene transfection without the need

for cellular pluripotency. In two studies, hepatocyte-like differentiation was achieved using Gata4, HNF1a and Foxa3 (106) or HNF4 with Foxa1, Foxa2 or Foxa3 (107).

Other approaches related to gene transduction have complemented the usual differentiation protocols based in supplementation with growth factors with the transduction of hepatic genes to enhance the differentiation. Takayama *et al* (108) have obtained hepatocytes from hESC and iPSC by transducing Sox17, HEX and HNF4a at different time points.

For clinical use, iPSC share some of the problems showed by ESC therapies. In one hand, many protocols are hampered by inefficient differentiation and maturation that lead to low yield and heterogeneous cell populations in cultures. On the other hand the risk for teratoma formation may arise due to the presence of a few undifferentiated iPSCs. Therefore, further enriching hepatocytes using negative selection against pluripotent cells could be useful to avoid teratoma formation.

In contrast to ESC, as iPSC can be patient-specific, they can serve as a tool for *in vitro* disease modeling. They can provide a more relevant model system because their properties closely resemble those found in the patient's own system, i.e. they can be a model for a certain disease without the need of genetic manipulation or any kind of induction. These cells can be used as models to study the pathogenesis, disease mechanisms and possible cure for liver disorders.

Hepatocytes from adult stem cells

It has long been thought that the differentiation potential of adult stem cells is limited to their germ layer of origin, but recent studies have demonstrated that adult stem cells are more plastic than once believed. Therefore, transdifferentiating from other tissue cells or adult stem cells has also been a focus on the regenerative medicine field.

Hepatocytes from hematopoietic stem cells (HSC)

Hepatic and hematopoietic tissues maintain a close association throughout the lifespan of mammals. The embryonic liver is the major site of blood cell formation from mesodermal cells. After the major site of blood cell formation moves from the liver to the bone marrow toward the end of liver development, mesodermal cells originating from hematopoietic cells, remain in the liver and continue to be partially replenished from the bone marrow in adult animals. Many reports have found that both rodent and human HSCs can be induced to differentiate into hepatocytes *in vitro* and *in vivo*.

Most protocols for HSC differentiation into hepatocytes *in vitro* used a growing medium conditioned with growth factors and mitogens (HGF, FGF or OSM) and culture layers specific for hepatocyte growth such as matrigel. Although these studies showed transdifferentiation of some HSCs into hepatocytes, the reported percentage of hepatocytes derived from HSCs did not exceed 5% (109). Thus, HSCs exhibit a limited differentiation potential that make them non-optimal candidates for tissue regeneration purposes. *In vitro* differentiation has also been demonstrated with mouse hematopoietic cells. The reports showed hepatocyte-like cells with expression of several transcription factors and cytoplasmic proteins, including some of mature hepatocytes (110).

HSC have been extensively used in clinical trials related with liver disease. Although it remains unclear if the cells are able to transdifferentiate in vivo or they develop other functions, the clinical outcome of the patient is usually positive, with prolonged (up to 1 year) improvement of liver function after treatment with HSCs (111, 112). However, in certain circumstances, an unfavorable ability of HSCs to preferentially differentiate to myofibroblast-like cells was observed in a murine model of liver injury (113) and in the liver of cirrhotic patients (114) thus contributing to hepatic fibrosis.

Hepatocytes from MSC

MSC isolated from several sources have been proven to give rise to hepatocytes *in vitro* and *in vivo*. MSC from bone marrow (115, 116), adipose tissue (117) and umbilical cord(118, 119) have been derived to hepatocyte-like cells with relative success. The first report generated hepatocytes from MSCs of mixed origin (from umbilical cord blood and bone marrow). The stem cells were cultivated in the presence of FGF and basic FGF that was replaced by a mixture of HGF and FGF (115).

BMMSC

The formation of hepatocytes from bone marrow-derived MSC was induced either with endothelial growth factor (120) or with sequential stimulation with HGF, FGF4, OSM and Dex (121). A DNA microarray analysis of *in vitro* differentiated human bone marrow MSC cultured in an hepatogenic medium, has shown up-regulation of several liver-specific genes (122).

BMMSC-derived hepatocytes can effectively rescue immunodeficient mice from lethal fulminant liver failure induced by toxin and can provide engraftment up to 5% of the recipient liver (123).

Clinical trials with MSC are less frequent than with HSC. However there are some encouraging results already published. An autologous BMMSC transplantation was safe for 53 patients with

liver failure caused by hepatitis B. It had favorable short-term efficacy and played important roles in repair after acute liver injury as well as improved disease condition and mortality (124). In other phase I and II clinical trials, injection of autologous bone marrow MSCs into the peripheral or portal vein resulted in a significant improvement in liver function as reflected by the restoration of normal levels of hepatic production of albumin, creatinine and bilirubin, and the absence of complications and acute side effects (125, 126).

Umbilical cord mesenchymal stromal cells (UCMSC)

Campard *et al.* (127) showed for the first time a 3-step method to induce UCMSC differentiation to hepatocyte-like cells with success. They used a bundle of factors including: EGF, bFGF, HGF, ITS, OSM and DEX and observed cell markers' changes towards hepatocytes characters. Other studies cultured UCMSC in medium with HGF, bFGF, DEX, insulin and sodium selenite during 16 days and then transferred them into OSM contained medium. After 28 days culture, the differentiated UCMSC showed hepatocyte-like morphology and expressed several liver-specific markers like: ALB, AFP, CK19, tryptophan 2,3-dioxygenase (TO) and G6P at both gene and protein levels. Furthermore, these cells exhibited hepatocyte-specific functions, including albumin secretion, low-density lipoprotein (LDL) uptake and urea production.

Experiments in vivo showed that after transplantation of undifferentiated UCMSC in liver, the engrafted cells expressed human hepatic markers such as albumin and AFP (127) and reduced the formation of liver fibrosis (128).

Adipose mesenchymal stromal cells (ADMSC)

ADMSC from patients of different ages after incubation with specific growth factors (HGF, FGF1 and FGF4) have been shown to acquire expression of various liver-specific markers in an adherent monoculture condition (116). Other studies have compared the ability of BMSC and ADMSC to differentiate into hepatocytes (129). The results show a similar protein expression pattern and comparable differentiation potential, demonstrating that ADMSC can express drug-metabolizing enzymes such as CYP2E1 and CYP3A4. Another report shows that differentiated ADMSC can uptake LDL and produce urea (117). Experiments with immune deficient mice have shown that HLC from ADMSC maintained the expression of several hepatic proteins in vivo and induced expression of albumin and bilirubin (130).

DPMSC

To date only few publications have studied the ability of dental pulp stem cells to generate hepatic-like cells. Ishkitiev *et al.* (131) showed that progenitor cells of dental pulp have hepatic potential and are able to express AFP, albumin, HNF4α among other markers. The same group

also showed that SHED can differentiate with similar efficiency and that were able to undergo several hepatic functions (132).

Only one report of experiments with rodents is available to date. SHED differentiated into hepatocytes were transplanted into rats with acute liver injury or secondary biliary cirrhosis. There was engraftment of human cells and there were human hepatic markers in the animals' blood (133) with no fibrosis or malignancy found.

Hepatocytes from MAPC

MAPC were the first plastic cells found within adult bone marrow that gained the ability to undergo hepatic differentiation. Using combined exposure to FGF4, HGF, ITS and DEX MAPCs transformed into cells with morphological, phenotypic, and functional characteristics of hepatocytes. They were able to express CK19, AFP, CK18, HepPar-1, and CD26, and produce albumin, urea, and glycogen (134). In experiments with rodents, MAPC have also been differentiated into hepatocyte-like cells that expressed several hepatic genes such as AAT, AFP, ALB, GSC and were able to develop many hepatic functions as urea and albumin secretion, glycogen storage and detoxification (135).

Despite the promising results, the differentiation status of hepatocyte-like cells from adult sources has not been characterized as well as from the pluripotent stem cells. The percentage of hepatic lineage cells tends to be different when different markers are used and the nature of the rest of the population is usually not reported. The efficiency of hepatic differentiation from adult tissues is still insufficient for clinical application in bioartificial liver or cell transplantation, which might be improved by modifying culture conditions or adding various growth factors/cytokines. As DPPSC have a great potential of differentiation (74), we believe that they can be a good adult stem cell source to obtain hepatocyte-like cells *in vitro*.

OBJECTIVES

Main objective:

To obtain a population of hepatocyte-like cells differentiated from DPPSC.

Secondary objective:

To characterize DPPSC and to compare their genetic expression with other cells of interest in regenerative medicine, such as DPMSC or iPSC.

HYPOTHESIS

$H1_0$: DPPSC have the same genetic profile as other stem cells from the dental pulp

$H1_1$: DPPSC possess a different genetic profile when compared to other stem cells from the dental pulp

$H2_0$: DPPSC do not have the ability to differentiate into hepatocyte-like cells

$H2_1$: DPPSC have the ability to differentiate into hepatocyte-like cells

MATERIAL AND METHODS

Patient selection

DPPSC and DPMSC were isolated from healthy human third molars extracted for orthodontic and prophylactic reasons from 9 patients with ages comprised between 14 and 25 year old. All samples were provided after obtaining the informed consent from donors. All experiments were performed in accordance with the guidelines on human stem cell research issued by the Committee on Bioethics of the Universitat Internacional de Catalunya.

Isolation of DPPSC

Immediately after extraction, the third molars were washed using gauze soaked in 70% ethanol and then washed with sterile distilled water. An incision was made between the enamel and the cement using a cylindrical turbine burr while holding the tooth with upper incisor forceps. A fracture was made on the same line as the incision using two upper incisor forceps, and the two fragments of the tooth were placed in a Falcon tube with sterile 1X PBS. The samples were rapidly moved to the laboratory. Once placed in a laminar flow hood, the contents of the tube were poured into a Petri dish. The tissues were isolated from the dental pulp using a sterile nerve-puller file 15 and forceps. Next, a cellular separation was performed digesting the tissue from the divided pulp by adding collagenase type I (3 mg/ml) (Sigma-Aldrich) and incubating for 45 minutes at 37ºC in order to completely digest the pulp tissue. Afterwards, using an insulin syringe, a mechanical cellular separation was performed. The sample was then centrifuged for 10 min at 400 rcf to separate the medium from the cells. The cells were washed twice with sterile 1X PBS and centrifuged again for 10 min at 400 rcf. Finally, they were resuspended in their specific media and cultured in 650 ml flasks (Nunc) precoated with 100 ng/ml Fibronectin (Life Technologies) for 1h (136).

Expansion of DPPSC

The cells expansion medium for DPPSC consisted of 60% DMEM-low glucose (Life Technologies) and 40% MCDB-201 (Sigma-Aldrich) supplemented with 1X SITE Liquid Media Supplement (Sigma-Aldrich), 1X linoleic acid-bovine serum albumin (LA-BSA) (Sigma-Aldrich), 10^{-4} M L-ascorbic acid 2-phosphate (Sigma-Aldrich), 1X Penicillin-Streptomycin (PAA), 2% fetal bovine serum (FBS) (Sigma-Aldrich), 10 ng/ml hPDGF-BB (R&D Systems), 10 ng/ml EGF (R&D Systems), 500 ng/ml LIF (Millipore), Chemically Defined Lipid Concentrate (Life Technologies), 0.8 mg/ml BSA (Sigma-Aldrich) and 55 µM β-mercaptoethanol (Sigma-Aldrich). For expansion, cells were

grown in 650 ml flasks coated with 100 ng/ml hFN in 5% CO_2 and a humidified chamber. Cell density (80-100 cells/cm^2) was maintained by detaching cells with 0.25% Trypsin-EDTA (Life technologies) and re-plating every 36-48 h (136).

Isolation of DPMSC

As the other population obtained from the dental pulp and different of DPPSC is not well characterized, they will be designed with a general name, DPMSC, from now on. The isolation process is the same as for DPPSC except the final step, where DPMSC are resuspended in their expansion medium consisting of DMEM with 4.5 g/L glucose (Life technologies) supplemented with 10% FBS (Cultek) and 1X Penicillin-Streptomycin (PAA).

Expansion of DPMSC

For expansion, cells are grown in 650 ml flasks (Nunc) kept in 5% CO_2 in a humidified chamber. Medium was changed every 2-3 days and cells were passaged when confluence was about 90%.

EB generation from DPPSC

EB were generated by two techniques. The first one was performed by the hanging drop culture technique, i.e. cells are seeded in small drops of their expansion medium all across a non-adherent petri dish (Thermo) and they are kept upside down for several days.

The second method uses AggreWell™ plates (Stemcell Technologies), where cells are seeded in micropatterned wells followed by centrifugation at 300 rcf for 5 minutes.

ALP staining

For ALP staining, cells were fixed in a solution of 4% paraformaldehyde (Sigma-Aldrich) in dPBS (Sigma-Aldrich) for 15 minutes at room temperature. After extensive washes with dPBS, the cells were incubated in NTMT solution (10 mM NaCl (Sigma-Aldrich), 100 mM Tris-HCl (pH 9) (Sigma-Aldrich), 50 mM $MgCl_2$ (Sigma-Aldrich) and 0.1% Tween-20 (Sigma-Aldrich) for 5 minutes and then in NTMT solution supplemented with NBT (Nitro-Blue Tetrazolium Chloride (Sigma-Aldrich)) and BCIP (5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (Sigma-Aldrich)) in the dark for 10 minutes.

Transmission electronic microscopy (TEM)

A piece of the cell pellet measuring 1 mm³ was fixed in Karnovski solution, consisting of 4% formaldehyde (Sigma-Aldrich), 5% glutaraldehyde (Sigma-Aldrich) and 0.2 M cacodylate buffer (Sigma-Aldrich). After 48 hours, the samples were soaked in araldite. The ultra-fine sections were stained for contrast with citrate and then observed using an electronic microscope (Zeiss EM900)

Images of optical microscopy

All images of optical microscopy were taken from the OX.3040 Euromex binocular microscope for phase contrast and with the camera DC.10000c CMEX-10 digital 10 Mpix USB-2 CMOS.

Flow Cytometry

Multiple monoclonal antibodies were used: CD34 PE-Cy5 (eBioscience), OCT4 FITC (RD SYSTEMS), CD45 PE-Cy5 (BD), CD105 FITC (BD), CD146 FITC (BD), STRO-1 PE (BD Pharmingen), TRA-1-60 PE (eBioscience) NANOG FITC (BD Pharmingen), AFP (Abcam). For the analysis of control samples, the IgG1 fluorochromes of FITC, PE and PE-Cy5 (BD Pharmingen) were used. The suspension of cells (in PBS and 2% FBS) was incubated for 45 min at 4ºC in the dark. Later, the cells were washed twice with PBS and 2% FBS and centrifuged for 10 min at 400 rcf. Cells were resuspended in 1 ml of PBS and 2% FBS. All flow cytometry measurements were made using a FACScan cytometer and analyzed with the winMDI 2.8 program.

Teratoma formation

Eight-week-old immunodeficientmice (Samtako Bio Korea, Seoul, Korea) were anesthetized with diethyl ether. Fifty microliters of a DPPSC or DPMSC cell suspension ($4x10^7$ cells/ml) mixed with 50 µl of Matrigel (BD) were injected subcutaneously into the dorsal flank of the mice, who were then housed with free access to water and food under specific pathogen-free conditions. After 3 or 5 weeks, the teratomas were surgically dissected from the mice followed by fixation with 4% paraformaldehyde and 1.25% glutaraldehyde, and the samples were subjected to histological analysis. Specimens were embedded in paraffin, cut into 3 µm sections, and stained with hematoxylin and eosin (H&E).

Immunofluorescence

Cells were seeded in multichambered slides (Fisher Scientific). Samples were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature. For intracellular ligands, cells were permeabilized with 0.1 M Triton X-100 (Sigma-Aldrich) for 10 min. Slides were then incubated in PBS with 5% BSA for 30 minutes for blocking. After that, samples were incubated for 1 hour with the corresponding primary antibody: AAT (Abcam), ALB (Abcam) and CK19 (Abcam). Then they were washed with PBS three times and finally they were incubated with the secondary antibody (Abcam). Between each step, the slides were washed with 1% BSA (Sigma-Aldrich) in PBS (Life Technologies). Cells were examined using confocal fluorescence microscopy (Confocal 1024 microscope, Olympus AX70, Olympus Optical, Tokyo).

Hepatic differentiation with HGF and FGF4

The cells to be differentiated into hepatocytes-like cells were seeded on Collagen type I-coated 6-well plates (CellCoat®) at a density of $5x10^4$ cells/cm^2. The medium used consisted on 60 % DMEM low glucose (Life technologies) with 40% MCDB (Sigma-Aldrich) and supplemented with 1X ITS (Sigma-Aldrich) and 0.25X LA-BSA and 0.1 mM of ascorbic acid 2-phosphate (Sigma-Aldrich), 1X Penicillin and Streptomycin (ATT), HGF (R&D)(10ng/ml) and FGF4 (10ng/ml) (R&D). Medium was changed every 2 or 3 days for 21 days (134).

Hepatogenic differentiation (2D)

The protocol for hepatogenic differentiation has been very variable during the experiments performed, but the most optimal for DPPSC is based on a the protocol used by Agarwal *et al*.(89) and is described as follows. The cells to be differentiated into hepatocytes-like cells were seeded on Collagen type I-coated 6-well plates (CellCoat®) at a density of $5x10^4$ cells/cm^2 and placed in RPMI medium (Mediatech) supplemented with GlutaMAX and penicillin/streptomycin and containing 0.5% defined foetal bovine serum (FBS; HyClone) and 100 ng/ml Activin A (R&D Systems). Three days post-induction, the medium was refreshed using the same RPMI-based medium with 100 ng/ml Activin A but replacing FBS by KOSR 2%. After 2 days, cultures were refreshed with the same medium. 2 days later, the medium was changed by RPMI medium supplemented with GlutaMAX and penicillin/streptomycin, and containing 2% KOSR, 10 ng/ml FGF-4 (R&D Systems), and 10 ng/ml HGF (R&D Systems). Three days later the cells were switched to minimal MDBK-MM medium (Sigma-Aldrich) supplemented with GlutaMAX and penicillin/streptomycin and containing 0.5 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich),

10 ng/ml FGF-4, and 10 ng/ml HGF. After another 3 days, the cells were switched to complete hepatocyte culture medium (HCM) supplemented with SingleQuots (Lonza) and containing 10 ng/ml FGF-4, 10 ng/ml HGF, 10 ng/ml Oncostatin M (R&D Systems), and 10^{-7} M dexamethasone (Sigma-Aldrich). Differentiation was continued for another 9 days for a total of 22 days of differentiation. At each stage, the medium was refreshed every 2-3 days.

Endoderm induction with different factors

The protocol was equivalent to that of Hepatogenic differentiation for the first 5 days (2D) but the medium was supplemented with either 50 ng/ml of Wnt3a, 50 ng/ml BMP4, both Wnt3a and BMP4, 10 ng/ml FGF4 or 10 ng/ml bFGF.

Endoderm induction in different conditions

If not stated otherwise, the protocol was equivalent to that of Hepatogenic differentiation (2D). The experiments where fibronectin was used, the cells were seeded in pre-coated plates with 100 ng/ml Fibronectin (Life Technologies) for 1h. For the experiments without FBS, the cells were cultured in 2% KOSR for the first 3 days instead of 0.5% FBS.

Co-culture

In the co-culture with HepG2 cells, DPPSC cells were cultured as stated in the Hepatogenic differentiation (2D) protocol and immediately cell inserts with 20.000 HepG2 cells/cm^2 were placed in the wells with their corresponding medium. HepG2 were kindly provided by Dr. Miguel Barajas (University of Navarra).

3D Culture in the Cell Carrier Glass Scaffold

For 3D culture we used the Cell Carrier 3D glass scaffold (Orla Protein Technologies). The glass scaffold were placed in 24-well plates and treated with 2% matrigel for 1 h. Cells were seeded then in the wells at a density of 25,000 cells per cm^2.

Sandwich overlay

Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Life technologies) was used as sandwich overlay. After cells were plated on Geltrex, medium was replaced by 50 µl/cm^2 of Geltrex, generating a sandwich. Plates were placed at 37% for 30 minutes to let solidification of the overlay. Then, fresh medium was supplied to the cells.

Scanning electronic microscopy (SEM)

For SEM, samples were fixed in 2.5% glutaraldehyde (Ted Pella Inc.) in 0.1 M Na-cacodylate buffer (EMS, Electron Microscopy Sciences, Hatfield, PA) (pH 7.2) for 1 hour on ice. After fixation, the samples were treated for a further 30 minutes with 1% osmium tetroxide (OsO4) for 1 hour. The samples were dehydrated in serial solutions of acetone (30–100%) with the scaffolds mounted on aluminium stubs. The samples were examined with a Zeiss 940 DSM scanning electron microscope.

Immunocytochemistry

Cells were fixed with ThinPrep® CytoLyt® Solution for 30 min at room temperature followed by 5 min centrifugation at 5,000 rpm. The supernatant was discarded and the pellet was solubilised again with CytoLyt (Thinprep) Solution. Then, we centrifuged at 3,000 rpm for 5 minutes and the pellet was solubilised now with PreservCyt (Thinprep) Solution for 15 min. Cellular collection was performed by a ThinPrep 2000 processor. The microscope slides were fixed for at least 30 min or for a maximum of 16 h in ethanol 96º. After being washed twice with distilled water for 5 minutes, the slides were then blocked in 0.5% hydrogen peroxide/methanol for 10 minutes. The immunostaining was performed using a Leica MaxBond autostainer following the standard protocols given by the manufacturer. All products if not specified are from Novocastra. Washing steps were 3 for 1 min each with bond wash solution. The antigen retrieval procedure was performed according to Bond™ heat standard protocol (ER1) using citric buffer with pH 6 for 30 min at 95°. The antibodies were AFP clone C3 and AAT (Abcam). Cells were incubated for 15 min at room temperature. After that, 3 washing steps for 1 minute each with Bond wash solution. Then, slides were incubated with the post-primary for 8 min. followed with 3 washing steps of 2 min each with bond wash solution. Next, slides were incubated in Polymer AP for 8 min followed by 2 washing steps of 2 minutes each with bond wash solution and 1 washing step of 1 minute with destilled water. After that, the Mixed Diaminobenzidine Refine was applied for 10 minutes and then for 5 more minutes. After 3 washing steps with distilled water for 1 minute each, samples were counterstained with water hematoxylin for 5 minutes and washed with distilled water and wash bond solution, respectively.

PCR

Total RNA was isolated using Trizol Reagent (Life technologies) according to the manufacturer's recommendations. Between 1 µg and 2 µg of total RNA with a ratio 260/280 between 1.8 and

2.1 was used for the subsequent steps. The cDNA was synthesized using the Transcriptor First Strand cDNA synthesis Kit (Roche). Real-time PCR was performed using the FastStart Universal SYBR Green Master (Roche). Values of gene expression were normalized using GAPDH. A list of the primers used for real time-PCR experiments are listed in Supplementary table 1. For regular PCR TopTaq MasterMix kit (Qiagen) was used. Total liver RNA (Life technologies) or Fetal liver RNA (Clontech) were used as a control in several occasions. Cell lysates from iPSC were kindly supplied by Dr. Miguel Barajas. Primers used are described in Table IV.

Gene	Forward	Reverse	Size (bp)
AAT	TCGCTACAGCCTTTGCAATG	TTGAGGGTACGGAGGAGTTCC	142
AFP	AGGGTGTAGCGCTGCAAACGA	TGTTCCTGGCCTTGGCAGCAT	142
ALB	CCTTGGTGTTGATTGCCTTTGCTC	CATCACATCAACCTCTGGTCTCACC	308
CAR	CCGTGTGGGGTTCCAGGTAG	CAGCCAGCAGGCCTAGCAAC	278
CK19	GAGGAAATCAGTACGCTGAG	GTTTCTGCCAGTGTGTCTTC	323
CYP3A4	GCCTGGTGCTCCTCTATCTA	GGCTGTTGACCATCATAAAAG	187
CYP7A1	AGGACGGTTCCTACAACATC	CGATCCAAAGGGCATGTAGT	194
FOXA1	GCCTACTCCTCCGTCCCGGT	CCGGGGTCATGTTGCCGCTC	112
FOXA2	GCGACCCCAAGACCTACAG	GGTTCTGCCGGTAGAAGGG	162
G6P	TCAGCTCAGGTGGTCCTCTT	CCTCCTTAGGCAGCCTTCTT	291
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	87
GATA4	TCCCTCTTCCCTCCTCAAAT	TTCCCCTAACCAGATTGTCG	156
GATA6	AATGACTCCAGAACAACAACTGGG	CTCCCTCCAGTCCCATCAGC	111
HNF4	ACTACATCAACGACCGCCAGT	ATCTGCTCGATCATCTGCCAG	103
HNF6	CTTAGCAGCATGCAAAAGGA	TGCGTTCATGAAGAAGTTGC	211
LIN28	GGAGGCCAAGAAAGGGAATATGA	AACAATCTTGTGGCCACTTTGACA	97
MIXL1	CAGAACAGGCGTGCCAAGTC	TTCCAGGAGCACAGTGGTTGA	94
MYC	ACAGAAATGTCCTGAGCAATCACCT	GCCAAGGTTGTGAGGTTGCAT	73
NANOG	CAGCCCCGATTCTTCCACCAGTCCC	CGGAAGATTCCCAGTCGGGTTCACC	391
OCT4	GACAGGGGGAGGGGAGGAGCTAGG	CTTCCCTCCAACCAGTTGCCCCAAAC	144
PXR	GGACCAGCTGCAGGAGCAAT	CATGAGGGGCGTAGCAAAGG	190
SOX1	AAGATGCACAACTCGGAGATCAG	TGTAATCCGGGTGTTCCTTCAT	133
SOX17	CTTTATGGTGTGGGCCAAAG	TTGTAGTTGGGGTGGTCCTG	186
SOX2	GGGAAATGGGAGGGGTGCAAAAGAGG	TTGCGTGAGTGTGGATGGGATTGGTG	151
TERT	CCTGCTCAAGCTGACTCGACACCG	GGAAAAGCTGGCCCTGGGGTGGAG	446

Table IV. Primers used for PCR amplification

Hepatic Biochemical Analysis of supernatants

Hepatic enzymatic profile (ALP, ALT, AST and GGT) was analysed from supernatants of the different samples. The activity was measured by four specific colorimetric detection kits (Linear

Chemicals) following the manufacturer's instructions. The specific compound used for following the kinetics of reaction were the following: for the GGT it was measured the formation of 3-carboxy-4-nitroalinine at 410nm, for the AST and ALT it was measured by the oxidation of NADH at 340 nm and for the ALP by the formation of 4-Nitrophenol measured at 405 nm.

Cytochrome P450 3A4 Metabolic Activity Assay

Cytochrome P450 (CYP) 3A4 enzyme activity assay was assessed by measurement of luciferase activity with the P450-Glo CYP3A4 assay (Promega), according to the manufacturer's instructions. Differentiated cells were treated with rifampicin (25µM) for 48 h, added fresh every 24 h. They were incubated at 37°C fresh medium with 50 µmol/L Luciferin PFBE and with or without erythromycin (5µM) for 30 minuts. Undifferentiated DPPSCs were used as negative control. After the incubation time, 50 µL of medium were transferred to a 96-well plate and mixed with 50 µL of luciferin detection reagent to initiate the luminescent reaction. After 20 minutes of incubation at room temperature, the luminescence was measured with a luminometer (Biotek).

Albumin Assay

Conditioned medium was collected over from equivalent numbers of cells. The albumin secretion was measured by using an Albumin Fluorescence Assay Kit (Fluka) following the manufacturer's instructions. Briefly, first a calibration curve was done with different human albumin concentrations. These calibration samples were mixed with albumin blue 580 in 2-propanol diluted in buffer solution and then the fluorescence was measured in a spectrofluorometer (λex=600 nm, λem=630 nm). Then, differentiation samples were mixed with the same reagents and the fluorescence was measured under the same conditions. Results were extrapolated to the calibration curve.

Periodic Acid–Schiff Staining for Glycogen Accumulation

Cells were fixed in 4% formaldehyde for 15 minutes at room temperature. After 2 washing steps with PBS, cells were incubated for 5 minutes in 1% periodic acid (Sigma-Aldrich) then washed with distilled water, and incubated with Schiff's reagent (Sigma-Aldrich) for 15 minutes. After a 10-minute wash in tap water, Karazi's hematoxylin counterstain was performed for 90 seconds minutes and washed with abundant water.

Short-chromosome genetic hybridization

Short-CGH technique was developed as described previously in Rius M. et al. (137) (n=13 for DPPSCs and n=18 for differentiated hepatocyte-like cells), analizing single cells from a homogeneous DPPSC culture.

Statistical analysis

Results are reported as an average ± standard error of the mean (see figure legends for specific details regarding the number of biological replicates, independent experiments and technical replicates). Statistics were performed using the Statgraphics XVI software. The methods used were two-tailed Student's t-tests and ANOVA for multiple factors. Values with $p<0.05$ were considered statistically significant.

RESULTS

Characterization of DPPSC

Morphology, gene and protein expression of DPPSC

The morphology of DPPSCs and DPMSC is different and can be observed by optical microscopy. DPPSCs are small-sized cells with large nuclei and low cytoplasm content without the typical flat and elongated MSC appearance (Figure 6A-B). DPMSC are spindle-shaped cells and have a bigger size (Figure 6C-D). The difference in size of DPPSC and DPMSC can be quantified by using an electronic cell counter. Although the range is variable in both populations, most DPPSC have a diameter between 6 and 13.5 µm and DPMSC between 14 and 18 µm (Figure 6E).

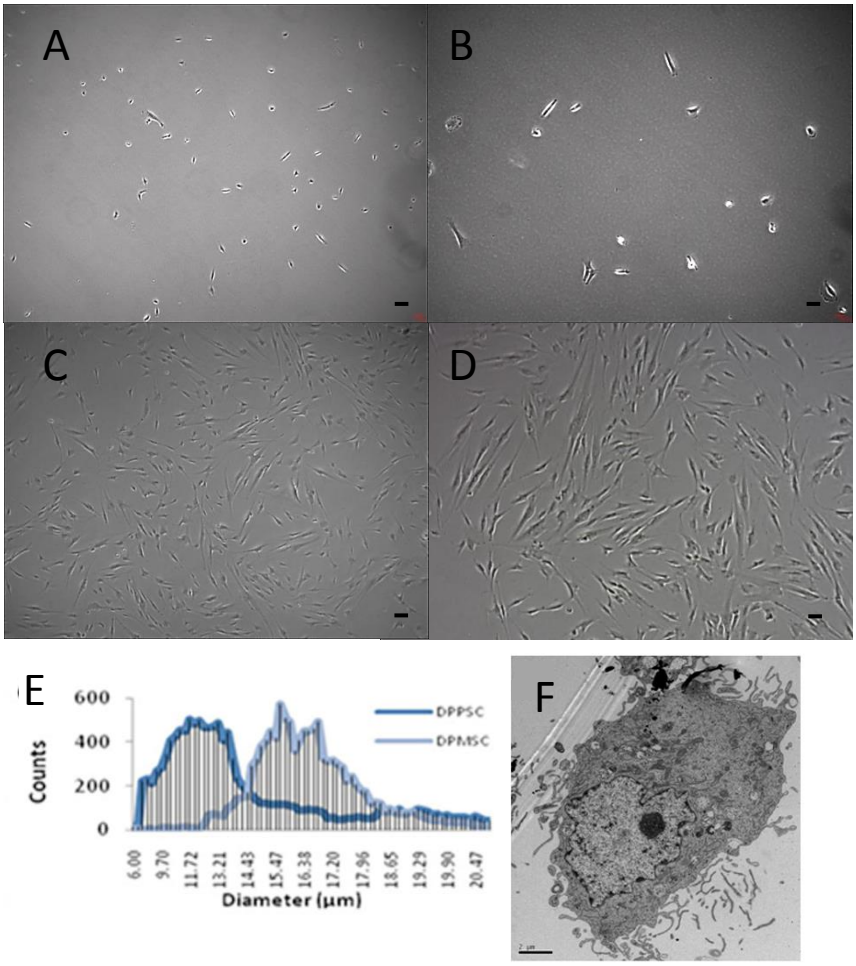


***Figure 6**: Morphology and size of DPPSC and DPMSC. **A-B:** Optical micrographs of DPPSC in culture. **C-D:** Optical micrographs of DPMSC in culture. **E**: Comparison of cellular size of DPPSC and DPMSC. **F**: TEM micrograph of DPPSC. Scale bars: 100 µm for A and C and 250 µm for B and D.*

A remarkable feature of DPPSCs is that they possess a large nucleus relative to the volume of the cytoplasm, which is a characteristic shared with ESC. The stability of the inner components of the cell is important to ensure their ability to give rise to descendants that maintain their properties, and DPPSCs showed no abnormalities in cell organs when checked by TEM (Figure 6F). The development of potential therapeutic strategies using stem cells depends on their ability to undergo large scale in vitro amplification that could imply genetic instability. DPPSCs exhibited a normal karyotype with no presence of any aneuploidy, polyploidy or any chromosome structural abnormality throughout several passages as checked by Short-CGH (Figure 7).

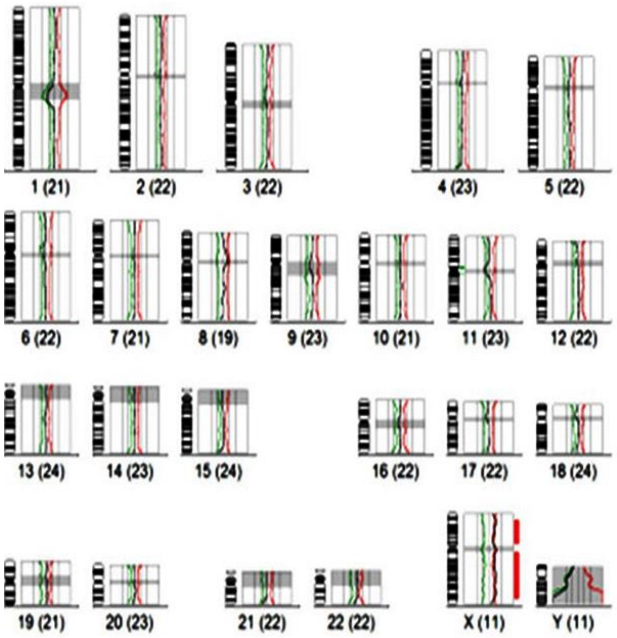


***Figure 7**: Representative result of Short-CGH from DPPSC.*

Aiming to characterize our cells we analyzed them by flow cytometry and we found that they were CD105+ (90.77%), CD146low (13.17%), CD45- (0.02%), CD34- (0.06%), STRO1low (4.42%), TRA1- (0.00%), OCT4+ (62.98%) and NANOG+ (22.96%). Pluripotent marker detection (OCT4 and NANOG) was carried out using non-labeled primary antibodies from mice and then anti-mouse secondary antibody in order to minimize false positives in the protein detection. In order to confirm that DPPSCs expressed simultaneously different embryonic markers, we performed a FACS analysis with double staining for OCT4 and NANOG. The results showed that 19.55% of the cells were double positive (Figure 8).

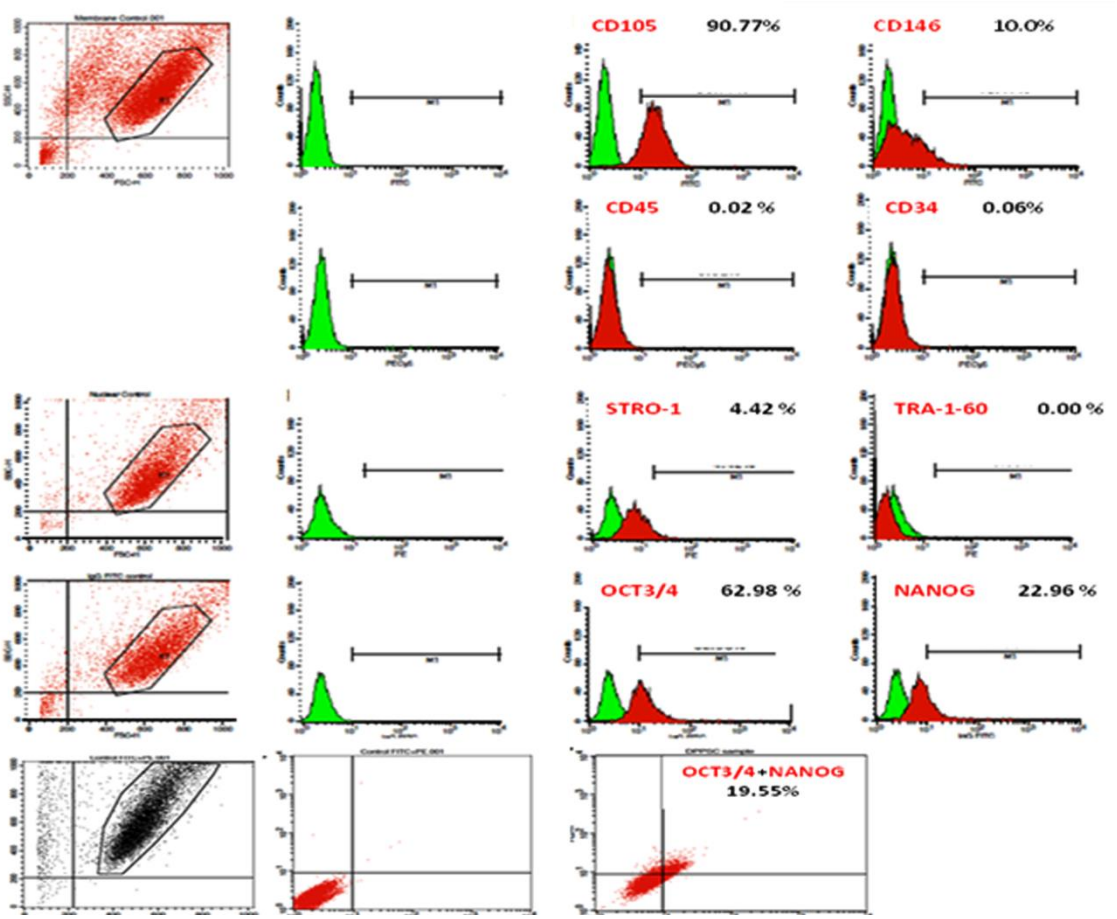


***Figure 8**: Protein expression checked by flow cytometry in DPPSC.*

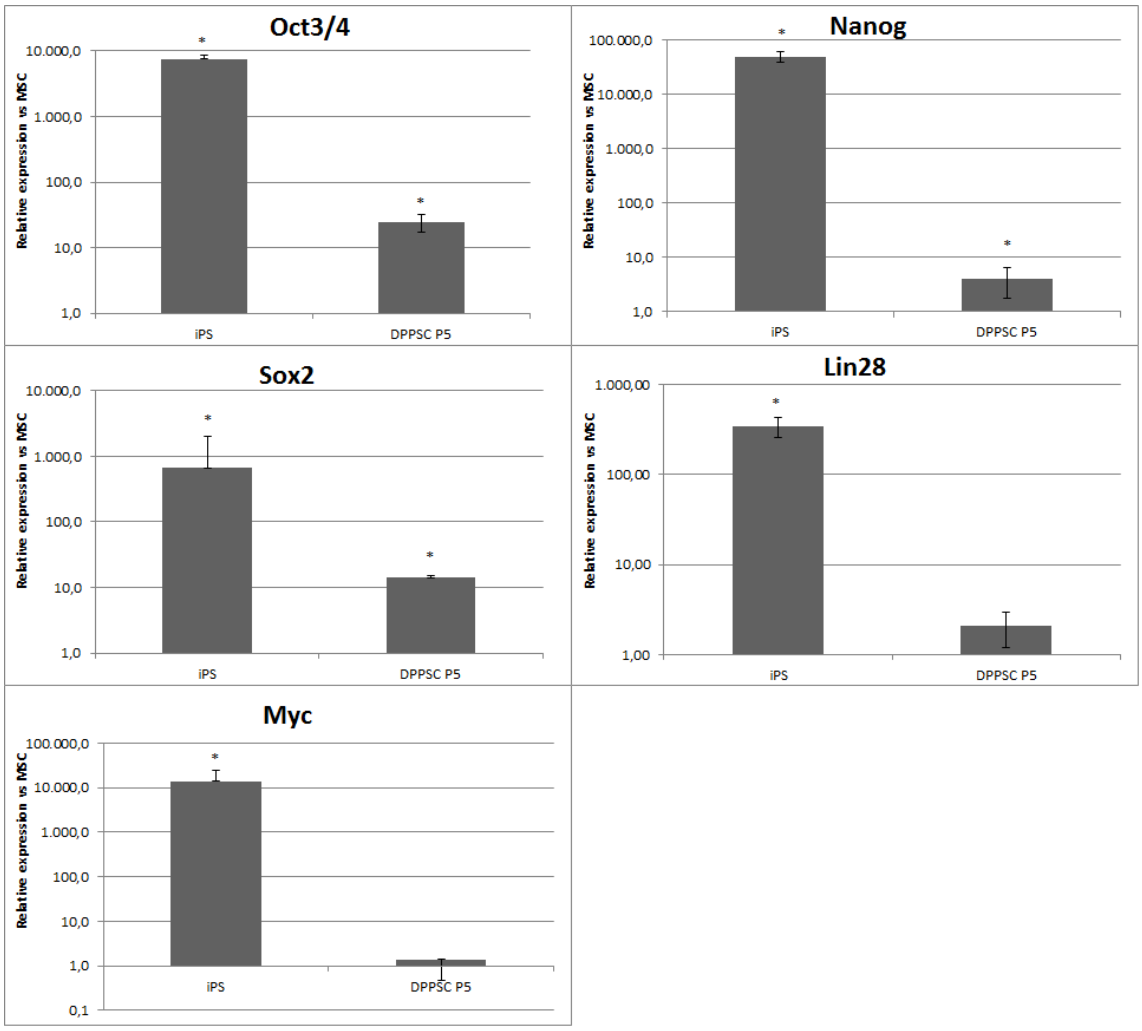


***Figure 9**. qRT-PCR comparing expression of pluripotency markers in DPPSC and iPSC with respect to DPMSC that were used as a control and set to a valor of 1. GAPDH was used as endogenous control. * p<0.05 in respect to all other samples (n=3)*

We performed several qRT-PCRs in order to analyze the expression of those embryonic markers in DPPSC and to compare them with mesenchymal cells as well as with iPSC. Our results showed that at P5, DPPSC have a higher expression of OCT4, NANOG and SOX2 compared to DPMSC of the same passage. Regarding the expression of other related genes, Lin28 and Myc are not evidently different in both populations (Figure 9).

Assessment of pluripotency

The pluripotency of DPPSCs was assessed *in vivo* by teratoma formation. The injection of DPPSCs into nude mice resulted in the formation of structures that contained tissues derived from all three embryonic germ layers in 3 out of 5 mice. In contrast, when DPMSCs obtained from the same donor sample were injected, no teratoma formation was observed. Staining with H&E showed the formation of multiple adult structures with origin in different embryonic layers: bone-like structures, chondrocytes, collagen structures, vessels and duct-like structures.

Immunohistochemical staining was performed in order to evaluate the expression of embryonic markers after 5 weeks. The results showed that some cells still retain the expression of embryonic markers meaning that they probably needed more time to form totally developed adult structures (Figure 10).

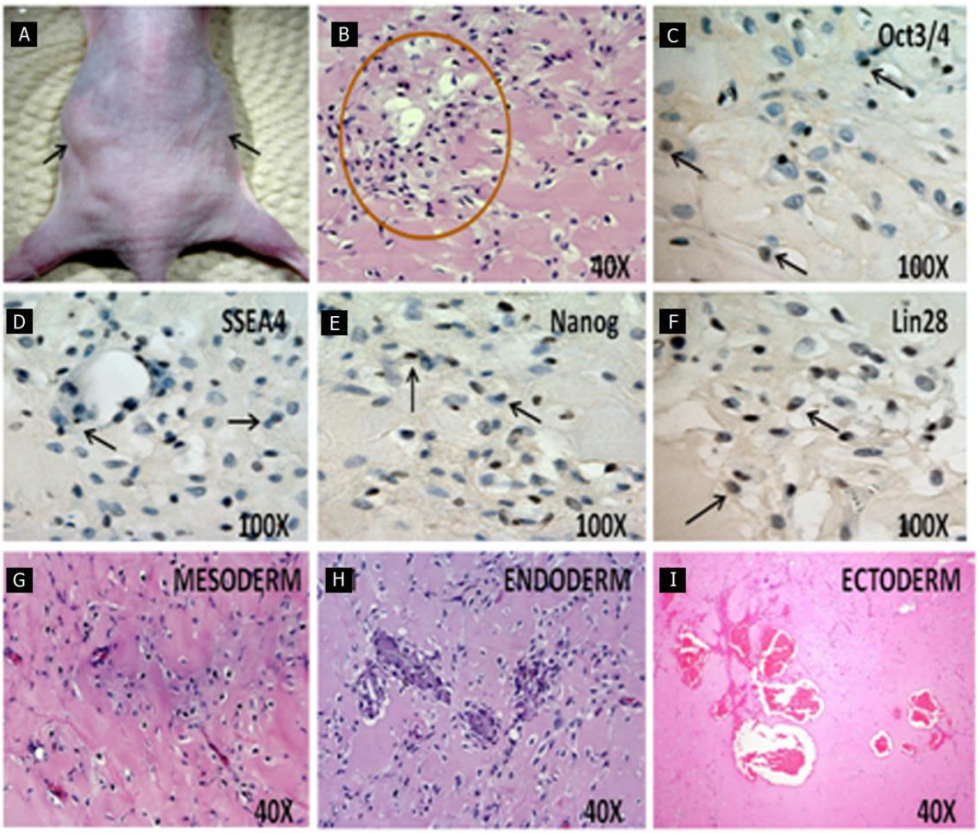


Figure 10. *Teratoma formation.* ***A****: Teratoma formed in mice injected with DPPSC (left) and DPMSC (right).* ***B****: Hematoxylin-Eosin stainin showing different cell types in one zone.* ***C-F****: Immunohystochemistry for OCT4, SSEA4, NANOG and Lin28.* ***G-I****: Hematoxylin-Eosin staining of teratoma showing tissues belonging to the mesoderm, endoderm and ectoderm germ layers.*

The ability of DPPSCs to form EBs was studied in two ways. First it was used the hanging drop culture and after a few days, EB were formed. However, the morphology was variable and the efficiency relatively low. Therefore we decided to use another method that consisted on using a micro-patterned culture plate and centrifugal force. After 5 days of culture, the morphology of EBs was evaluated by light microscopy (Figure 11A-B). EBs exhibited the typical spherical and well-limited appearance of EBs formed from ES cells. ALP staining showed however that the cells were in an undifferentiated state (Figure 11C). The EB continued to express embryonic markers such as OCT4 and NANOG at day 5, as observed by immunofluorescence (Figure 11D-E).

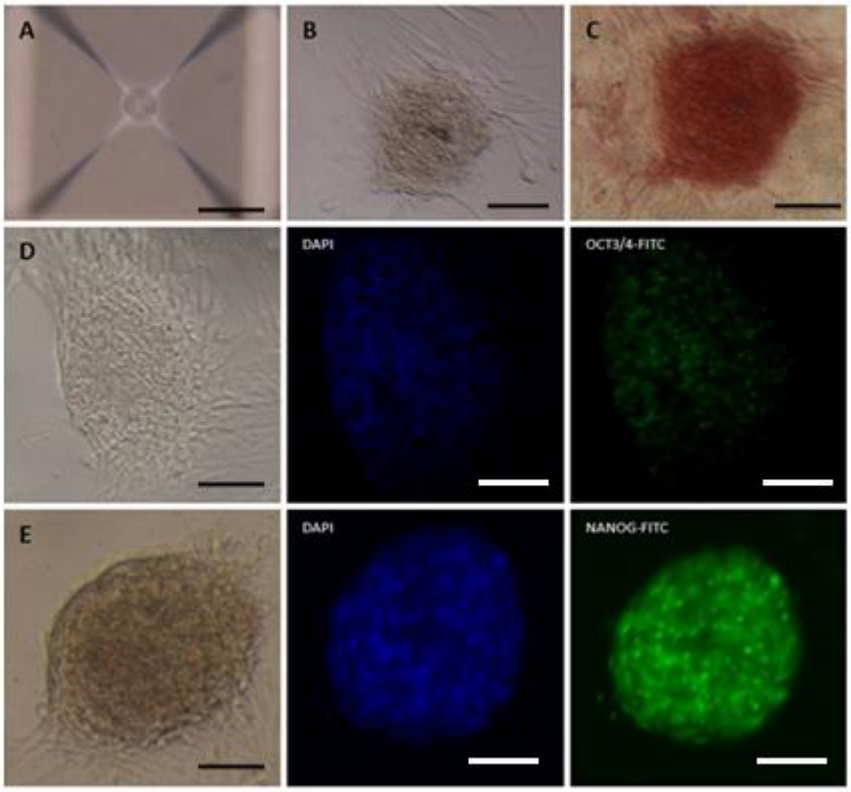


***Figure 11**. Embryoid body formation and genetic analysis. **A, B**: Morphology of DPPSC embryoid bodies examined by light microscope **C:** Alkaline phosphatase staining of DPPSC EB. **D** Immuno-phenotype checked by fluorescence microscopy shows the expression of OCT4 FITC: **E:** Immuno-phenotype checked by fluorescence microscopy shows the expression of NANOG FITC. Scale bars: 500 µm (A) and 50 µm (B-E).*

The expression of embryonic markers and lineage specific markers was studied by qRT-PCR Levels of OCT4 and NANOG were considerably high in DPPSCs when compared to MSC and tended to decrease when the EB were formed. However, this decrease in expression was not statistically significant. SOX2 had an important increase when EB were formed with levels higher than those of iPSC. DPPSC had only slightly higher levels than MSC. Lineage markers representative of the 3 embryonic layers such as Sox1, GATA 4, and MIXL1 underwent a massive increase in expression upon EB formation (Figure 12).

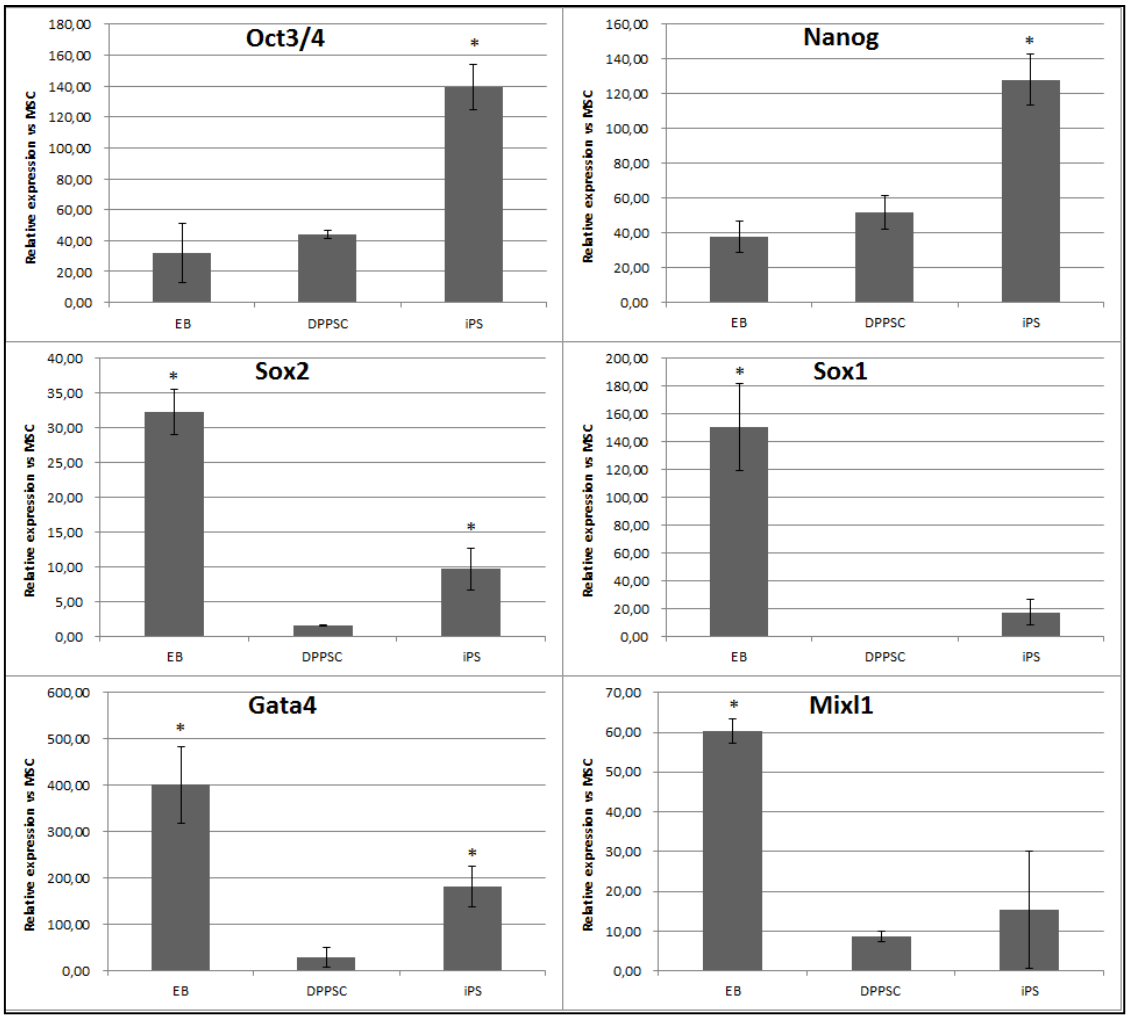


Figure 12. *qRT-PCR showing the expression of embryonic and linage-specific genes in DPPSC, EB from DPPSC and iPSC. GAPDH was used as endogenous control. Results are expressed relative to MSC expression. * p<0.05 in respect to all other samples (n=3).*

Interactions of DPPSC and DPMSC in culture

Due to the lack of a stringent selection when performing primary culture of DPPSC, we wanted to figure out if other populations were present in the culture of DPPSC at the same time. At first we thought that the different morphology (size and complexity) of the cells would allow us to to stablish a correlation between the size of the cells and the expression of CD73, OCT4 and NANOG. We thought that smaller cells should have a higher percentage of OCT4 and NANOG, while bigger cells should have a higher expression of CD73 (Figure 13). However, the results were not satisfactory as the peaks of expression overlapped. Therefore, we performed a cell sorting with CD73 as the selection protein as we had some previous data that showed that DPPSC did not express CD73 in contrast to MSC.

FSC	Oct3/4	Nanog	CD73
400-500	4.49	4.09	1.78
500-600	27.05	26.19	11.05
600-700	33.07	33.35	23.05
700-800	17.02	16.47	20.21
800-900	6.71	6.72	9.68
900-1000	3.48	3.67	3.33

SSC	Oct3/4	Nanog	CD73
200-300	7.13	7.73	1.39
300-400	23.24	22.34	12.81
400-500	25.12	25.59	19.07
500-600	15.19	14.89	15.41
600-700	9.24	8.82	9.14
700-800	4.32	4.42	5.01
800-900	3.94	3.33	2.78
900-1000	2.59	2.3	1.62

Figure 13. *Percentage of cells that express each marker in every range of size (FSC) and complexity (SSC).*

After that, we checked the genetic expression of both populations and saw that cells that were negative for CD73 expressed OCT4, SOX2 and Tert. However, the cells that were positive for CD73 did not express those markers. Therefore, we could consider that in culture there were, at least, two populations that could be distinguished by the expression of CD73, OCT4, SOX2 and Tert. Considering the previous information, we assume that the CD73+ population was from MSC and the $CD73^-$, $OCT4^+$, $SOX2^+$ and $Tert^+$ was the population of DPPSC (Figure 14).

We tried to continue culturing the cells after the cell sorter process but unfortunately they did not survive, probably due to the traumatic process of cell sorting.

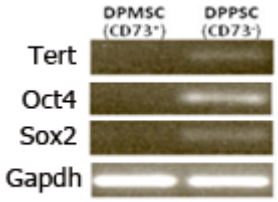


Figure 14*. Agarose gel showing the RNA expression of OCT4, SOX2 and Tert in cells* $CD73^+$ *and* $CD73^-$

In order to study whether the culture conditions were a key aspect for the maintenance of the different phenotypes between DPPSCs and DPMSCs, we cultured each type of cell in the conditions of the other one (medium and cell density). After 10 days changing the medium every 2 or 3 days we observed some phenotypic changes (Figure 15A). DPPSCs acquired a longer and flattered shape whereas some of the DPMSCs became smaller and with morphology resembling DPPSCs.

Changes were easier to identify in DPPSCs cultured in mesenchymal media than in the opposite way. We checked the expression of the embryonic markers that differed between the two cell types. We observed that DPPSCs cultured in mesenchymal medium lost the expression of NANOG, whereas the OCT4 levels only decreased. In the case of DPMSCs cultured in DPPSC medium, cells gained the expression of both OCT4 and NANOG (Figure 15B).

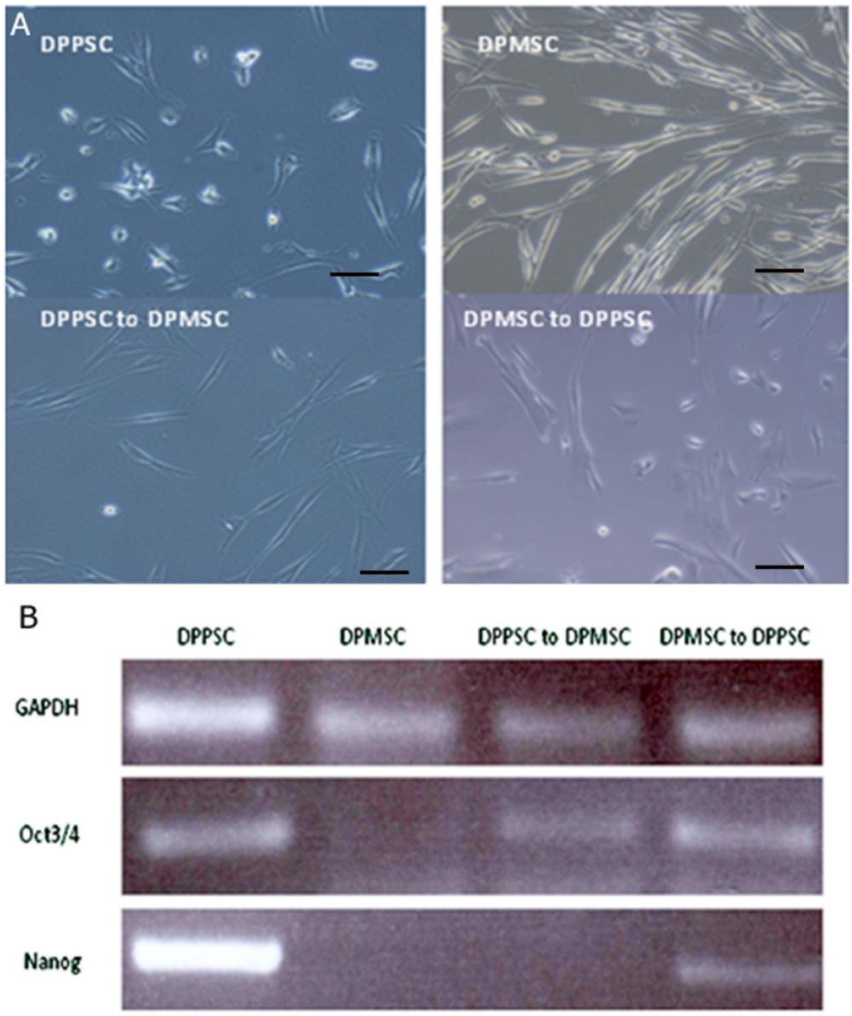


***Figure 15**. **A**: Changes in morphology in cells when culture conditions were changed**.** Scale bars: 50 µm. **B**: RT-PCR showing expression in cells when culture conditions were changed.*

Hepatic differentiation

The first approaches to hepatic differentiation with DPPSC were to test if different culture media were appropriate to carry out differentiation. We wanted to know if they could differentiate and in that case, what medium would be the most suitable.

Our first attempt to obtain hepatic-like cells was with the use of a simple medium based on the factors FGF4 and HGF as hepatic inductors (134). The results were not successful as only some irregular expression of some genes was obtained. Furthermore, cell death was very high at later stages and many times very little sample was available to examine (Figure 16-17).

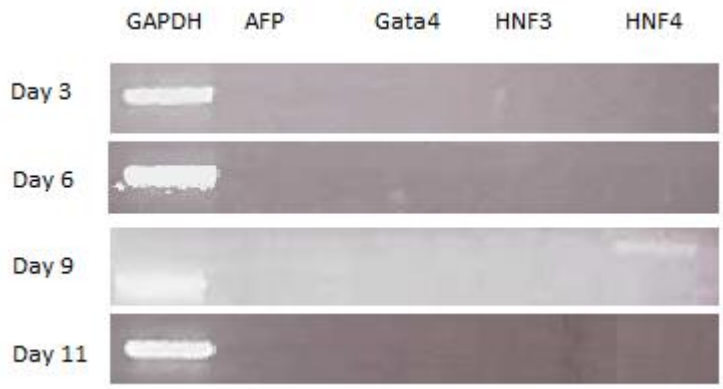


***Figure 16**. RT-PCR gene expression of hepatic markers during the differentiation of DPPSC*

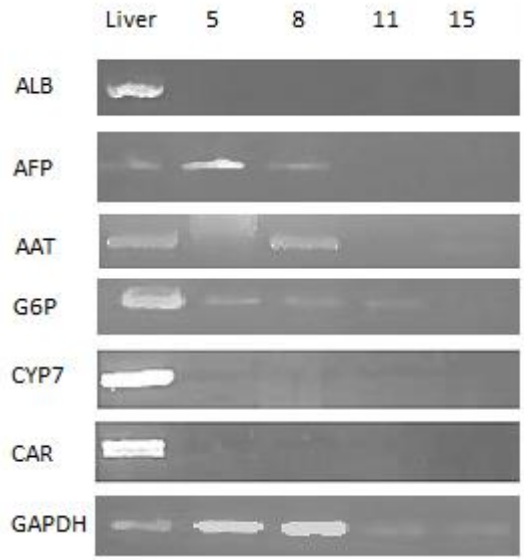


***Figure 17**. RT-PCR gene expression of hepatic markers during the differentiation of DPPSC.*

We realized that the first step to achieve was the formation of definitive endoderm, as it had been proven in several research articles that it was a key step when trying to obtain

hepatocyte-like cells. Then we addressed this by two different ways. The first one was by forming EB (80) from the cells and then differentiating from them. After 6 days, EB were able to enhance their expression of Gata6 and Gata4 (Figure 18). However, they did not express hepatic genes such as Albumin (not shown).

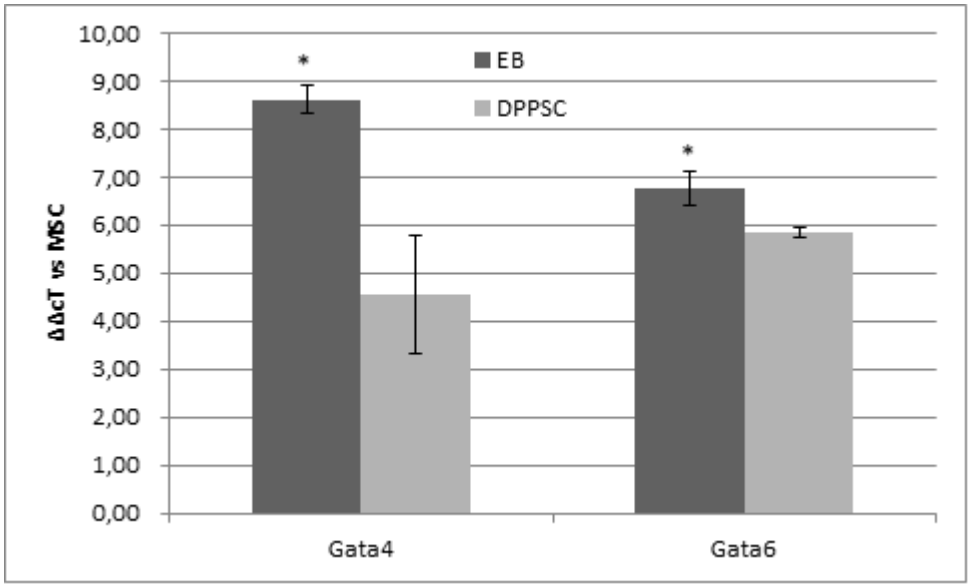


***Figure 18**. Changes in RNA expression of endoderm genes in DPPSC when they form EB. Samples were compared to MSC and GAPDH was used as endogenous control. * p<0.05 in respect to DPPSC. (n=2)*

We tried to plate these EB in adherent well-plates supplemented with regular DPSC growth medium and evaluate how cells developed. The cells survived with no complications, they started to migrate and populate the culture surface (Figure 19). With this information, we tried to strengthen their endoderm and hepatic fate by appliying different hepatogenic mediums to direct the differentiation through the use of cytokines. We compared different protocols published to determine if any of them was suitable for DPPSC (89, 95, 134).

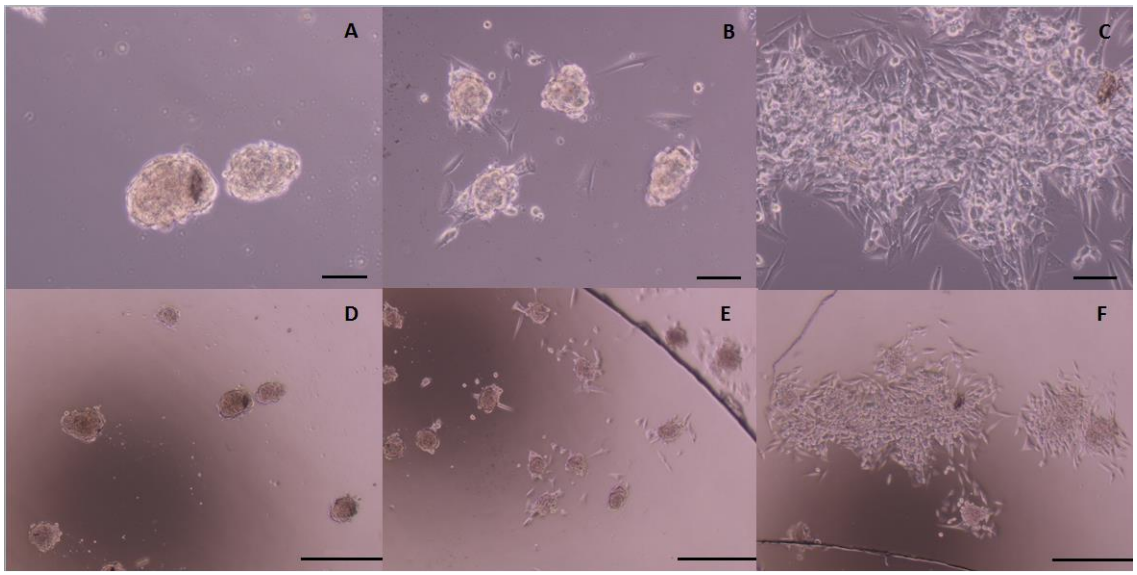


***Figure 19**: Changes in morphology of EB when they were re-plated in culture dishes at day 0 (**A** and **D**), day 1 (**B** and **E**) and day 5 (**C** and **F**). Size bars: 100 µm (A-C) and 500 µm (D-F).*

For that reason we used 3 different protocols, one based only in HGF and FGF4, another based in Activin A and Wnt3a and another based only in Activin A. Our results showed that the protocols that used Activin A were more effective than the protocol that did not use it, although the results were not very clear and it was hard to compare between them (Figure 20).

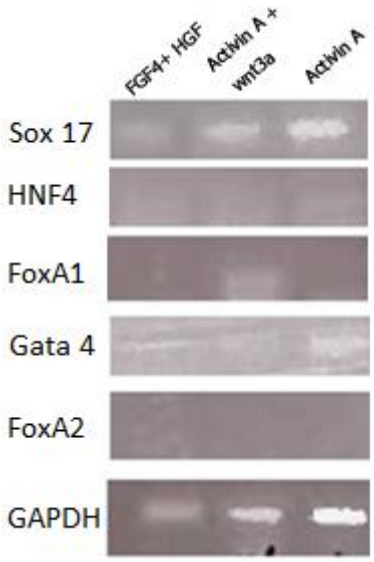


***Figure 20**: RT-PCR of hepatic genes in different differentiation conditions, after 7 days.*

Generation of definitive endoderm

Expression of endoderm markers was only present using the mediums 2 and 3, although was not homogeneous. The results suggested that Activin A was necessary for the obtainment of definitive endoderm with DPPSC, however we wanted to evaluate if other factors would enhance this conversion. Several pathways are involved in endoderm generation, so we drew our attention to some of them. We had already checked Wnt3a but we wanted to do it in a quantitative way. We also checked other cytokines such as BMP4, the combination of BMP4 and Wnt3a, bFGF, and FGF4. We checked the expression of Foxa1, Foxa2, Gata4 and AFP as markers of endoderm and early hepatic differentiation.

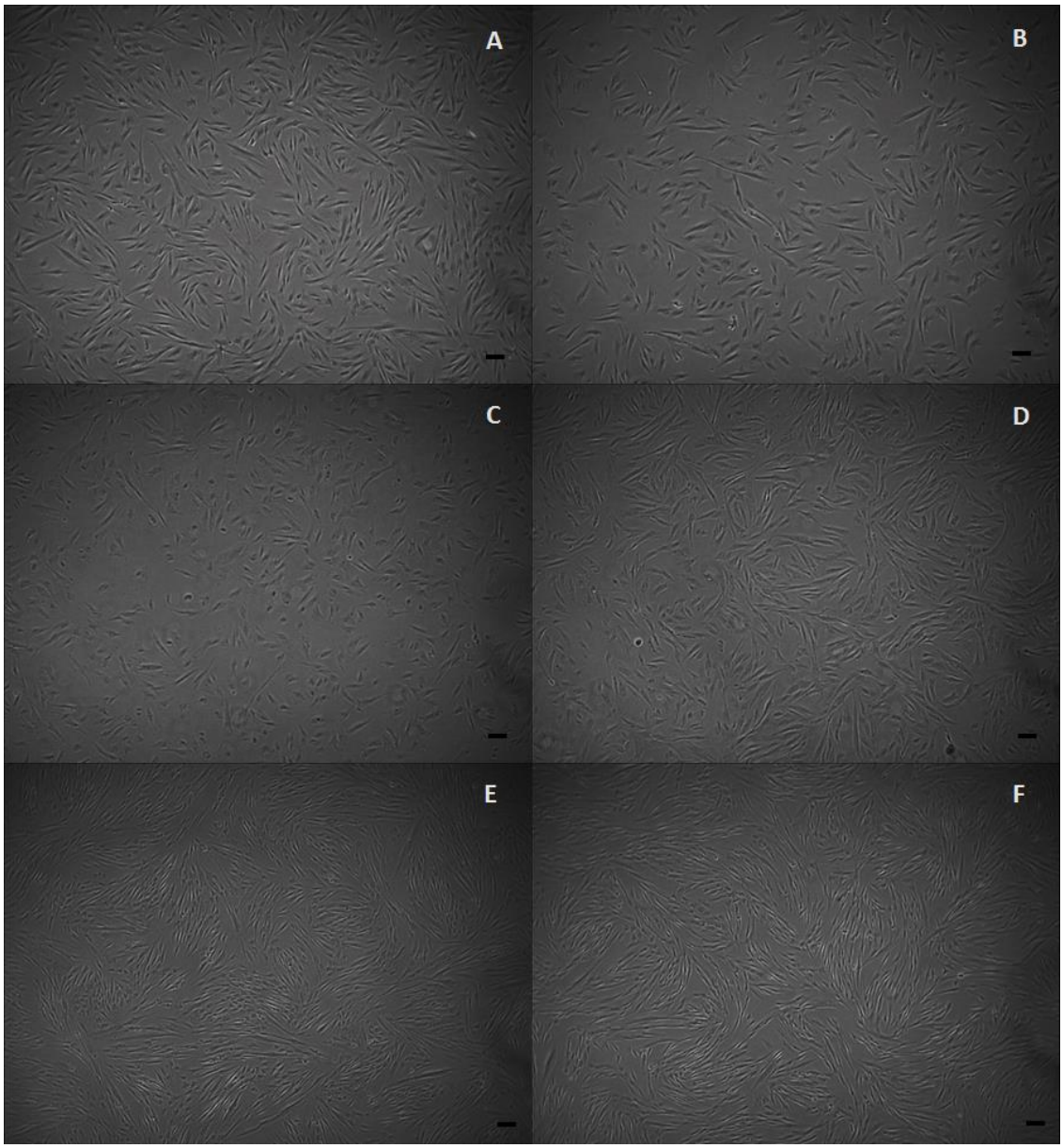


Figure 21. Morphological evaluation of the effect of different factors in the generation of endoderm after 5 days. Activin A was present in all of them. **A**: Activin A alone **B**: Wnt3a **C**: BMP4 **D**: Wnt3a + BMP4. **E**: bFGF. **F**: FGF4. Size bars: 100 µm

Morphological changes were detectable by optical microscopy. The group of Activin A alone, with Wnt3a and with Wnt3a and BMP4 had a similar morphology, but the group with Wnt3a had a lower cell growth. The group with BMP4 had a more uniform morphology with smaller size than the other ones. The group with bFGF and FGF4 acquired a more elongated shape (Figure 21). However, no conclusions can be obtained only from observation so we proceeded to analyse their genetic expression.

Regarding Foxa2, apparently the group with BMP4 was the one with a higher expression; however it did not have a significant difference in respect with the Activin A alone, and the Wnt3a supplemented group. These 3 groups were equally effective when compared to undifferentiated cells (Figure 22).

When looking to AFP expression, the group with only Activin A was the one with the highest expression in comparison with undifferentiated cells. Moreover, the addition of any other factor such as Wnt3a, BMP4 or FGF4 to the medium did not improve the differentiation and overall decreased the expression levels of AFP.

In the case of Foxa1, the treatment with Wnt3a was beneficial for the differentiation as it increased the level of expression of this gene. In any case, the group treated with BMP4 and with FGF4 were equivalent to that treated only with Activin A.

Regarding the expression of Gata 4, the group cultured with only Activin A as an inductor showed the higher expression but with levels statistically equivalent to those of the ones treated with Wnt3a and BMP4.

Overall, the addition of a growth factor such as Wnt3a or BMP4 together with Activin A in the first stages of differentiation had little to no impact on the differentiation after 5 days. It is remarkable that the addition of bFGF or Wnt3a + BMP4 apparently inhibited the inductor effect of the Activin A.

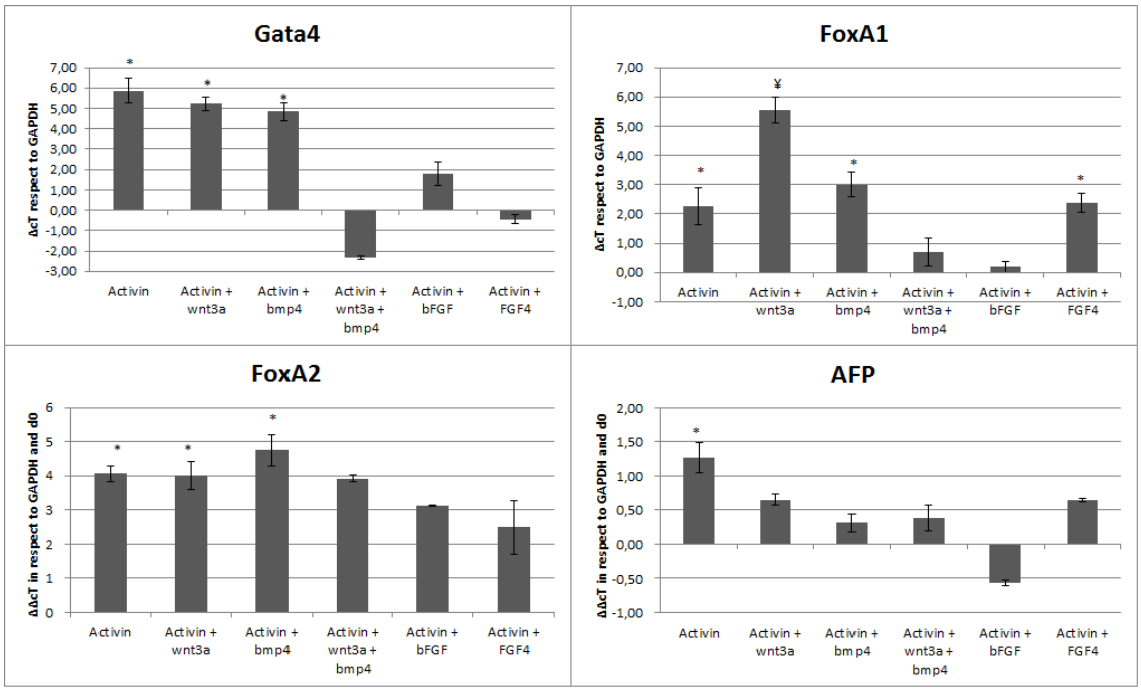


***Figure 22**. Quantitative expression of hepatic genes in different differentiation conditions, after 5 days. Expressed in ΔΔcT in respect to undifferentiated cells. GAPDH was used as endogenous control. *p<0.05 in respect to undifferentiated cells. ¥ p<0.05 in respect to all other sample. (n=4)*

After this, we wanted to further improve the differentiation conditions during the first 5 days of differentiation. We wanted to check other conditions in order to see their influence in the first days of differentiation. Therefore we carried out the differentiation in different conditions: the standard protocol in collagen I wells, in wells treated with fibronectin, in collagen without FBS during the 5 days, and with a co-culture with HepaG2 cells in cell inserts.

The evaluation of the morphology throughout the 5 days of differentiation showed some interesting information. Apparently, cells attached better to Collagen I than to Fibronectin, moreover, after 3 days there was more cell death in FN, -FBS and +HepaG2 compared to Collagen I alone. (Figure 23) At day 5, the cells in –FBS were the ones with a more evident apoptosis. However, the cells that were co-cultured with the HepG2 cell line grew in an exaggerated way and they acquired a more flat and elongated morphology and covered the whole surface of culture (Figure 24).

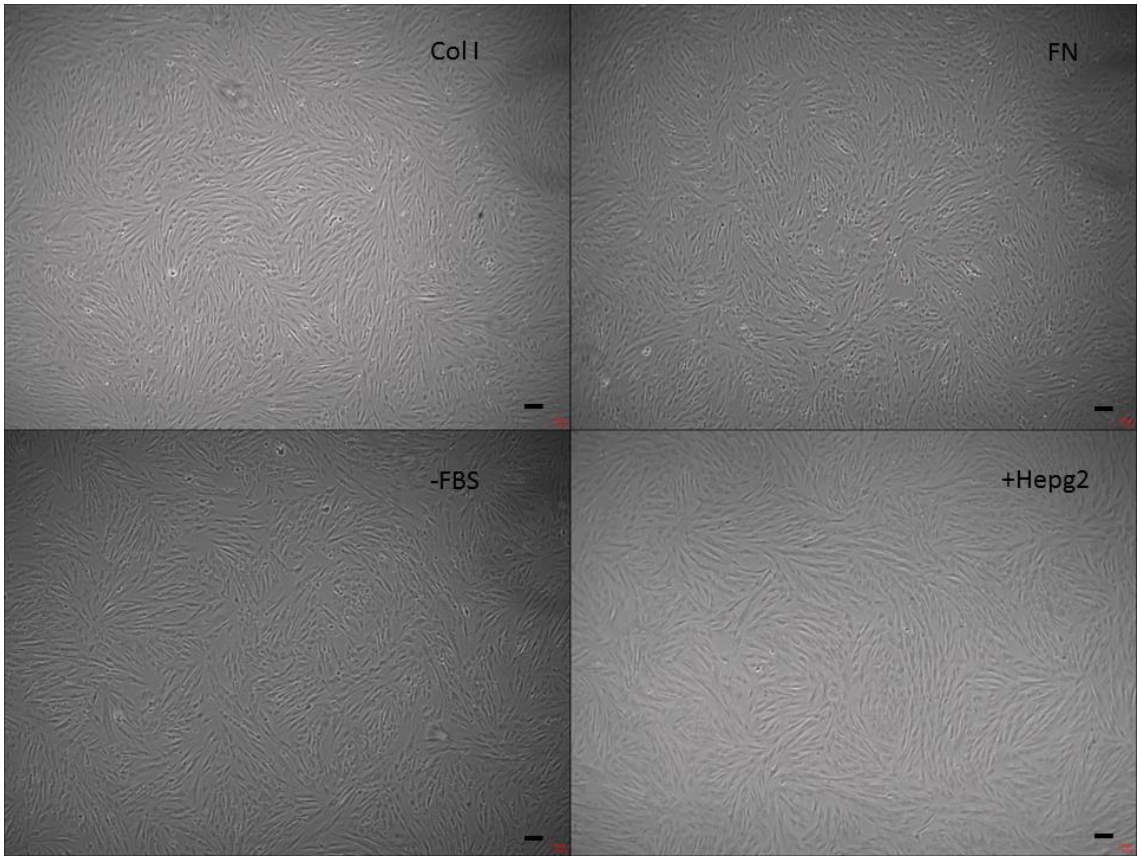


***Figure 23**. Morphology changes after 3 days of differentiated DPPSC in different conditions. Size Bars: 50 µm*

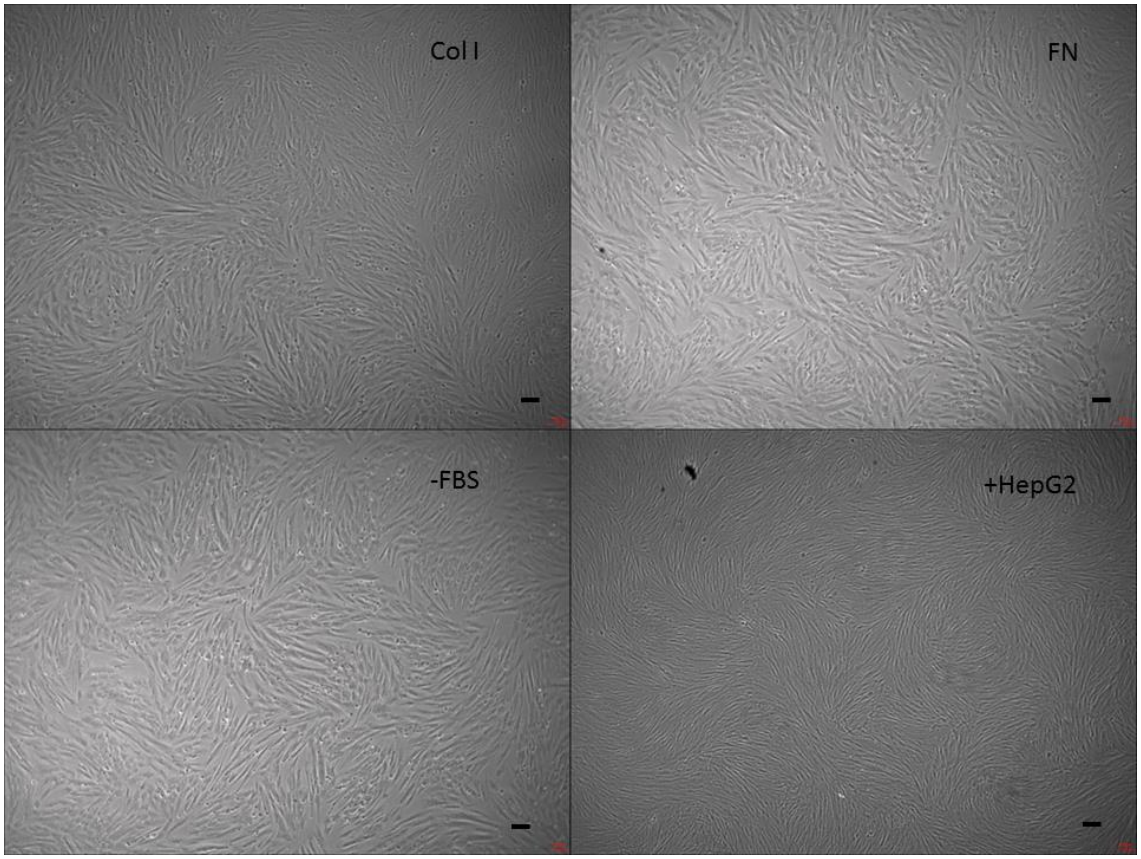


***Figure 24**. Morphology changes after 5 days of differentiated DPPSC in different conditions. Size Bars: 50 µm*

We performed a qRT-PCR to quantify the expression of Foxa1, Foxa2 and Gata4, genes that are expressed in the early stages of differentiation and compared them in the different conditions with respect to the standard protocol in collagen I.

The results showed that the differentiation carried out in fibronectin had no significant relevance when compared to collagen I. There was a slight tendency to express higher levels of Foxa1 and Foxa2 but not in a significant manner (Figure 25).

Regarding the presence of FBS during the three first days of the differentiation, the results demonstrate that its absence is detrimental for the formation of definitive endoderm as the expression of Foxa2 and Gata4 was downregulated in that condition (Figure 26).

Concerning the co-culture with the HepG2 cell line, the results of qRT-PCR confirmed the signs that were evident by the morphology changes of the cells. The cells had less expression of the three genes and especially the decrease in Foxa2 was statistically significant suggesting that the co-culture with the hepatoma cell line did not help the generation of endoderm (Figure 27).

With these results, we conclude that the differentiation carried out in Collagen I is apparently as effective as in Fibronectin, that cells needed FBS the first three days of differentiation and that the presence of HepG2 was not beneficial for the differentiation.

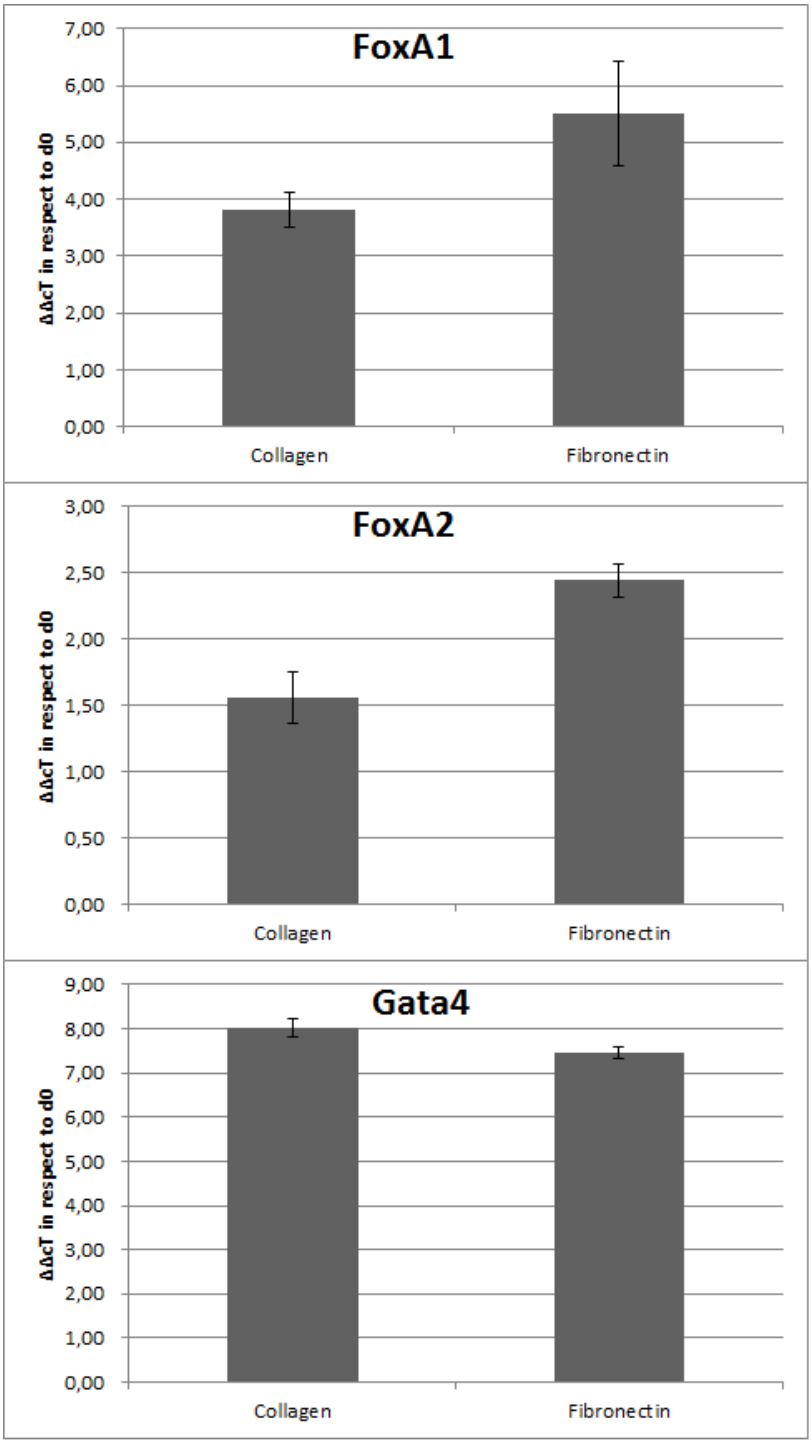


***Figure 25**. qRT-PCR expression of hepatic early genes in different surfaces, after 5 days. Expressed in ΔΔcT in respect to undifferentiated cells. GAPDH was used as endogenous control (n=2).*

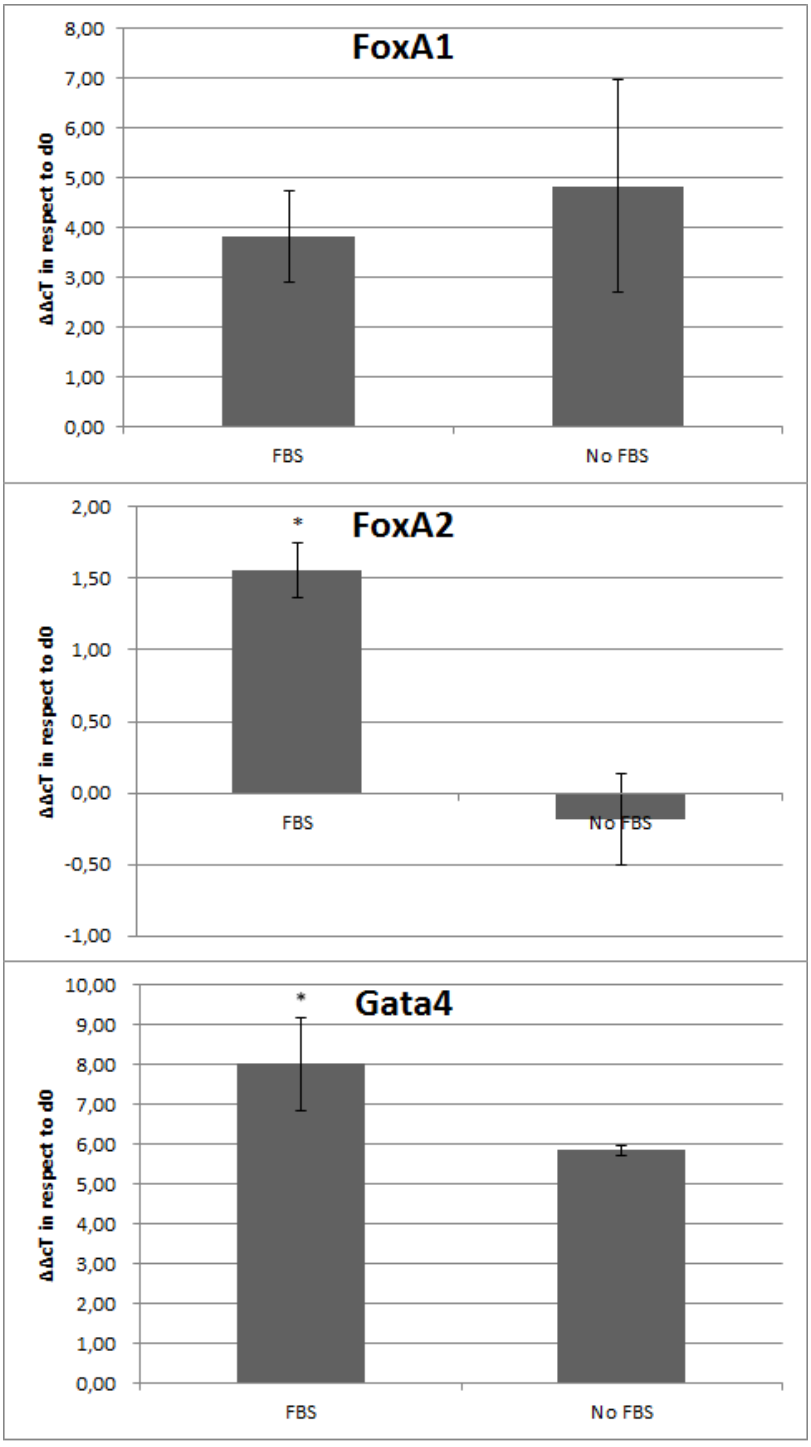


***Figure 26**. qRT-PCR of hepatic early genes with or without FBS, after 5 days. Expressed in ΔΔcT in respect to undifferentiated cells. GAPDH was used as endogenous control. *p<0.05 in respect to undifferentiated cells (n=2).*

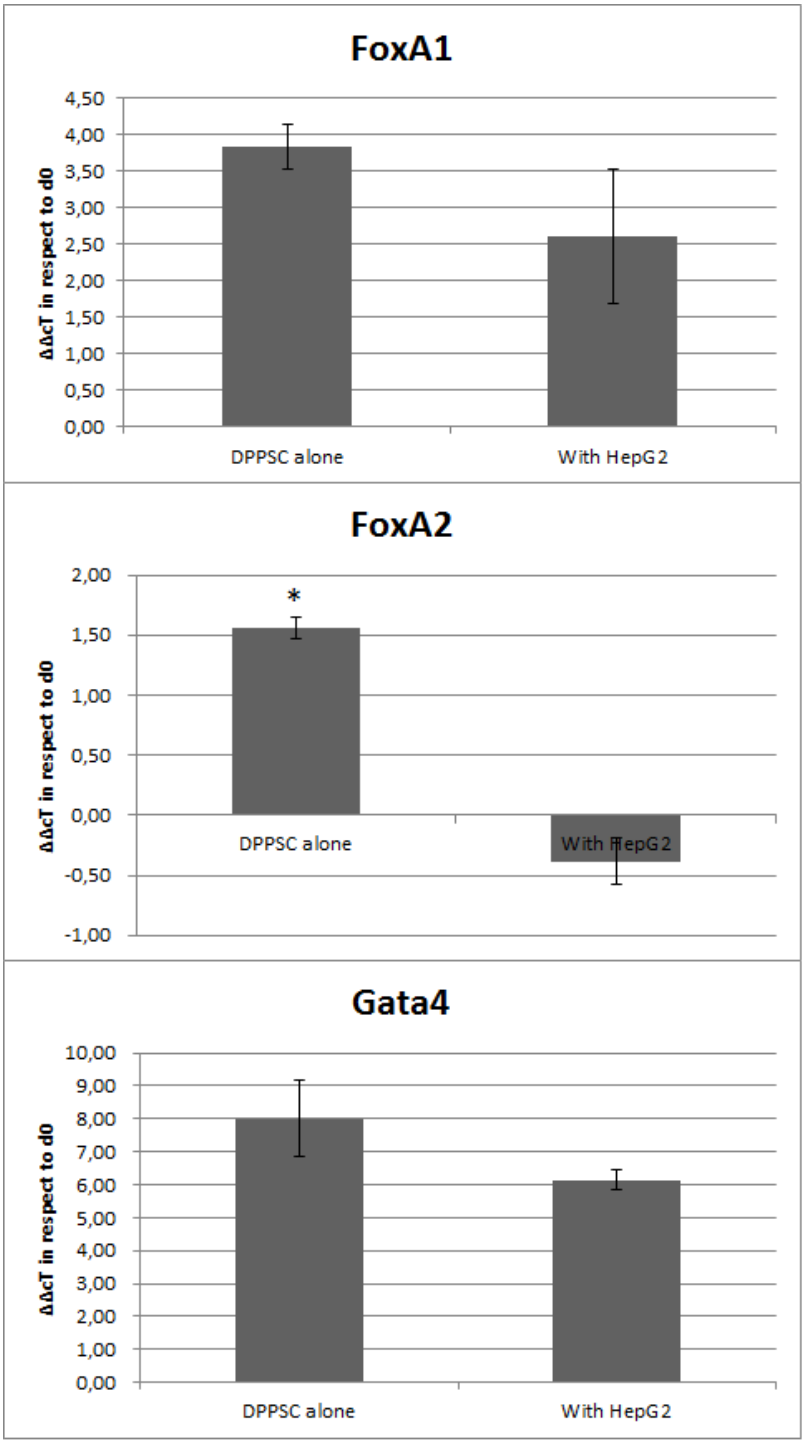
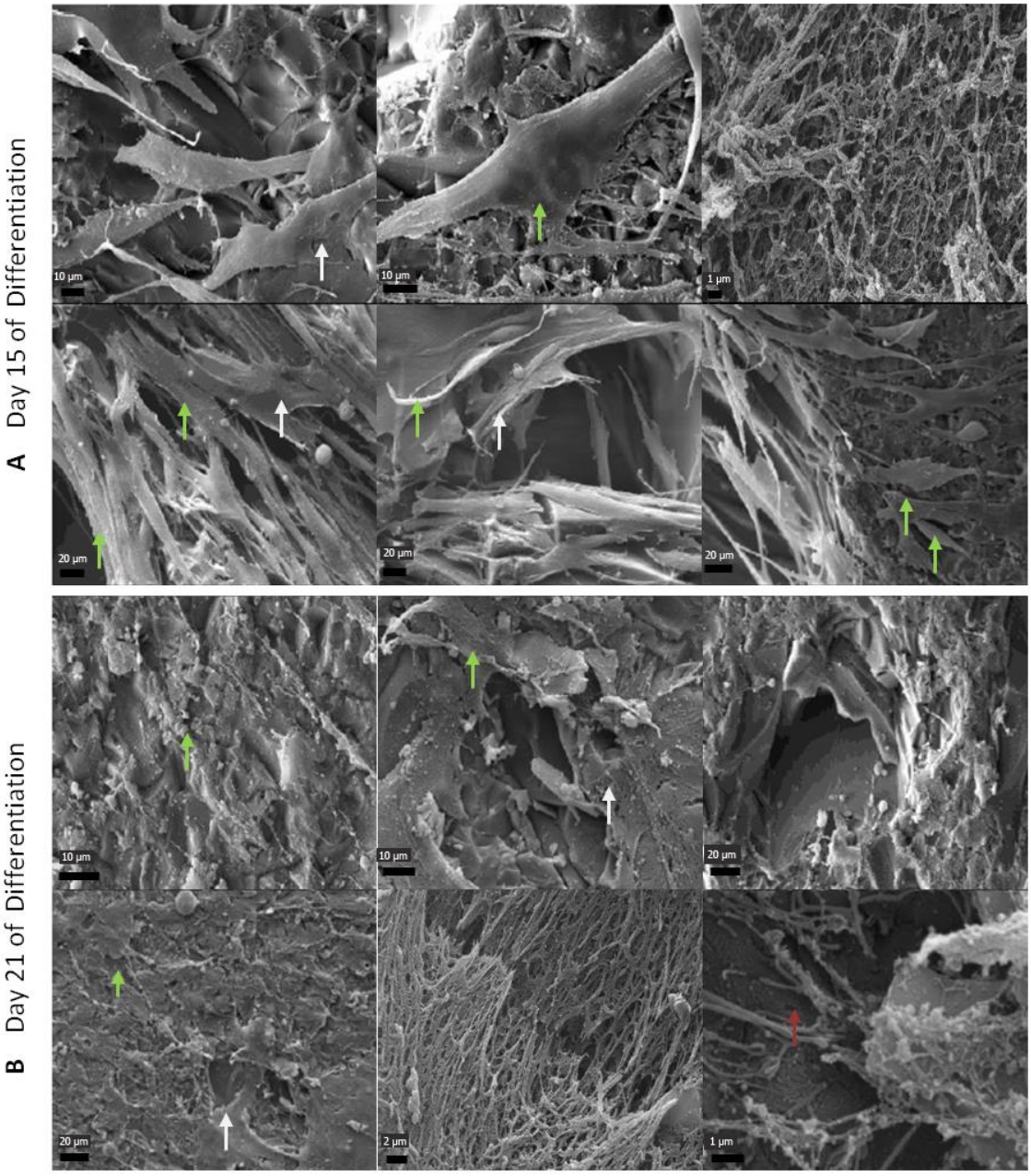


***Figure 27**. qRT-PCR of hepatic early genes with or without co culture with HepG2, after 5 days. Expressed in ΔΔcT in respect to undifferentiated cells. GAPDH was used as endogenous control. *p<0.05 in respect to undifferentiated cells. (n=2)*

Different approaches to achieve hepatic specification from the definitive endoderm

After obtaining definitive endoderm, we thought that exposure to HGF and FGF4 and subsequent exposure to OSM and Dex would be a good way to induce hepatic specification and maturation. As previous results were not very successful, we hypothesized that

introducing the use of 3D scaffold in our culture could improve the differentiation. It is known that contacts cell-cell and cell-surface are very important for the differentiation process, so we wanted to add that extra-element to increase the differentiation success. However, cells in 3D are harder to monitor by regular microscopy so we performed an analysis by SEM to check their evolution throughout the differentiation. Although hexagonal morphology, characteristic of hepatocytes, is hard to distinguish, some hepatic-like structures can be observed. Large and small pores with a fenestra-like appearance are shown (white arrow) in both time points, as well as the ultrastructure of sinusoidal endothelial cells (green arrow). At day 20, ultrastructure



of extracellular matrix can also be observed (red arrow)(Figure 28).

Figure 28**. SEM micrographs of DPPSC differentiated in a 3D glass scaffold after 15 days **(A)** and 21 days **(B).

When we checked the genetic expression of the cells throughout the differentiation we could see that the process was not optimal. The expression of AFP increased during the first 14 days of differentiation but at day 20 dropped to levels even lower than undifferentiated cells. Fully mature hepatocytes do not express AFP so this could be either the cells were completely differentiated or that they somehow dedifferentiated after the 14 days mark. The expression of ALB was irregular throughout the differentiation with similar levels of transcript after 7 and 21 days. CK19 had a peak of expression at day 7 and then decreased at following stages. HNF4 had a similar behaviour but the peak of expression at day 7 was less intense. HNF6 had also a peak of expression after only 7 days of differentiation, then decreased strongly at day 14 and finally increased a bit at the end of the differentiation. The cytochrome CYP7A1 had no expression after 7 days but after 2 weeks increased its expression despite the following fall at day 20 (Figure 29). The results show a big variability in the expression of several genes depending on the sample. When we performed regular RT-PCR the expression levels was equivalent in 3D and 2D (Figure 30).

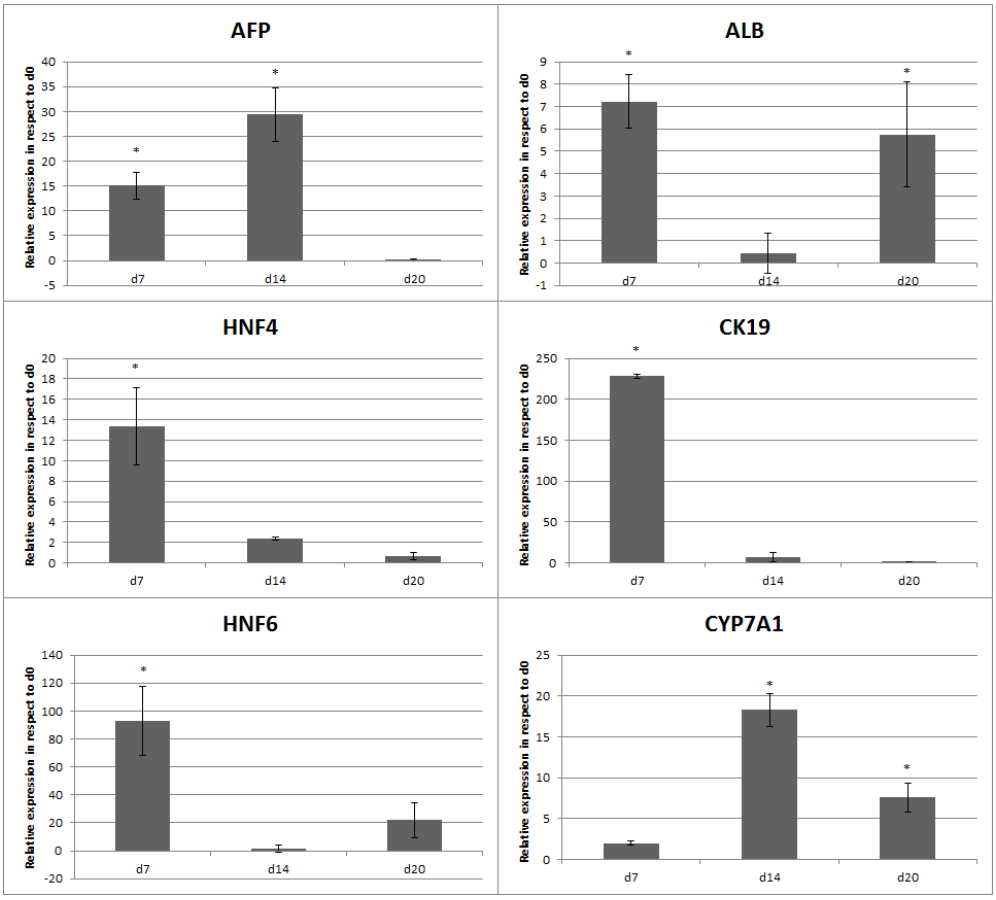


Figure 29. *Quantitative expression of hepatic genes in the 3D glass scaffold at different time points. GAPDH was used as internal control. Results are expressed in ΔΔcT in respect to liver RNA. *p<0.05 in respect to undifferentiated cells (n=3).*

These results showed that although apparently there was a good induction of endoderm after 7 days of differentiation, the following stages were not as good and a successful differentiation was not taking place in this 3D glass scaffold environment.

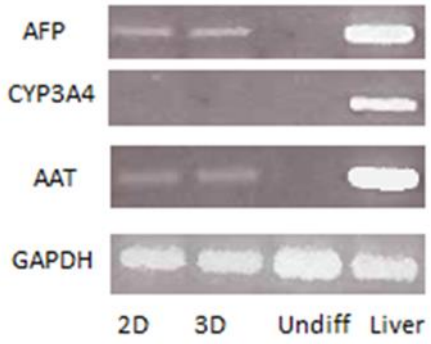


Figure 30*. RT-PCR showing expression of different hepatic genes in differentiated DPPSC both in 3D glass scaffold and 2D conditions.*

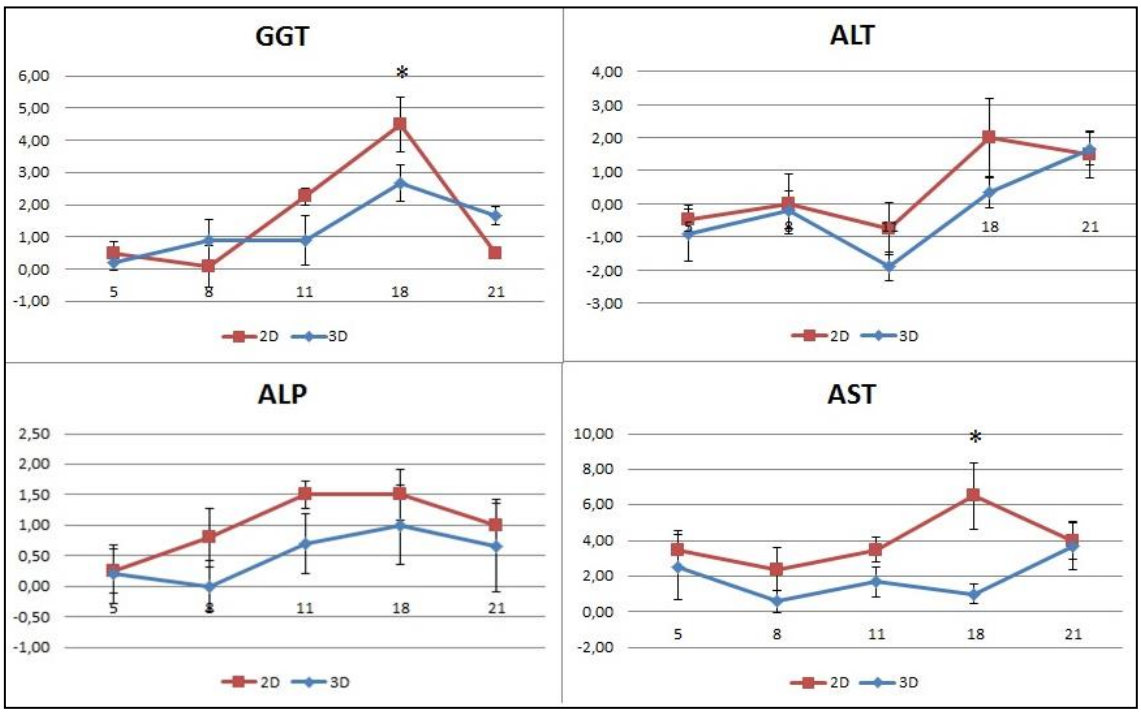


Figure 31*. Enzymatic activity of GGT, ALT, ALP and AST in supernatants of differentiated cells both in 2D and 3D conditions at different time points. Expressed in units of absorbance.*p<0.05 in respect to 3D (n=3).*

Another piece of data that suggests that 3D differentiation in the glass-scaffold was not successful regarding the measurement of activity of several hepatic enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gammaglutamyl transferase (GGT). The results showed a different behavior when comparing differentiation in 3D and in 2D. Regarding the ALP and ALT activity, the profile was similar in both cases but the results were higher in the 2D group, although in both cases the enzymatic activity was considerably low. For the AST activity, cells in 2D had a clear peak of activity after 18 days of differentiation whereas the cells in 3D had a regular profile at all stages with lower activity. In the case of GGT the profile was similar in both groups but cells in 2D had a peak of activity that almost doubled that of the 3D cells (Figure 31).

In addition to all these discouraging results, we could evidence that in several occasions after the differentiation ended we were not able to obtain as much biological samples as we desire in order to perform our posterior analysis when cells were cultured in 3D. In most cases we had around 25% and 30% cell survivability in 3D compared with 2D. These results made us think that the environment was not appropriate for the cells.

Therefore we focused on performing the differentiation in 2D environments and we wondered if the hepatic specification was possible in these 2D conditions. We checked the expression of several hepatic related markers after one week and two weeks (Figure 32).

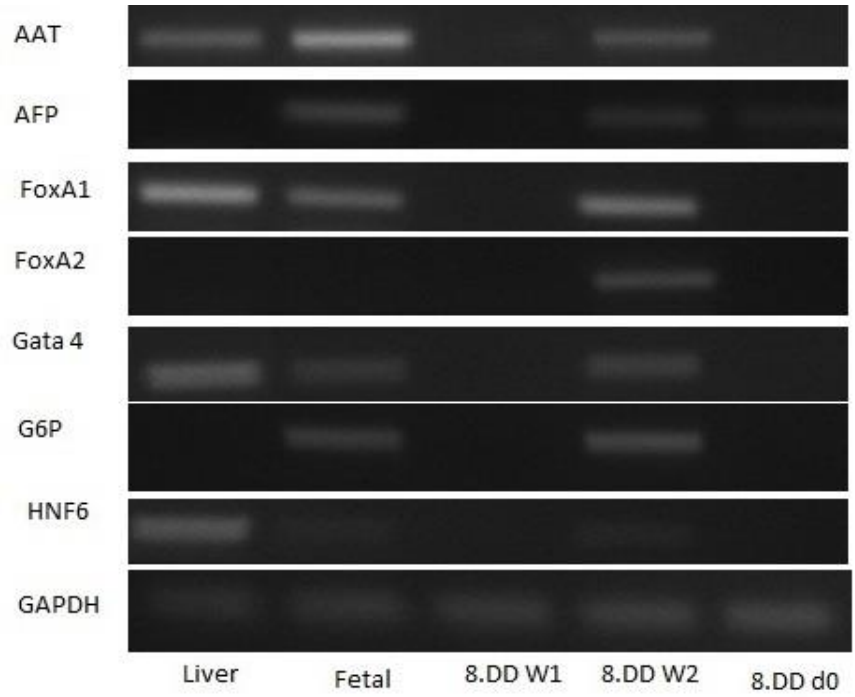


***Figure 32**. RT-PCR of hepatic-related genes of DPPSC differentiated after 7 and 14 days compared with undifferentiated cells and with positive controls.*

In order to test if we could improve the step to obtain definitive endoderm, we decided to expose the cells to Activin A for 2 more days and see if that could accelerate the first steps of differentiation. The results showed that cells were able to express early hepatic genes and endoderm genes such as AFP and Foxa1 in the first 7 days of differentiation while with the standard protocol they needed more days (Figure 33).

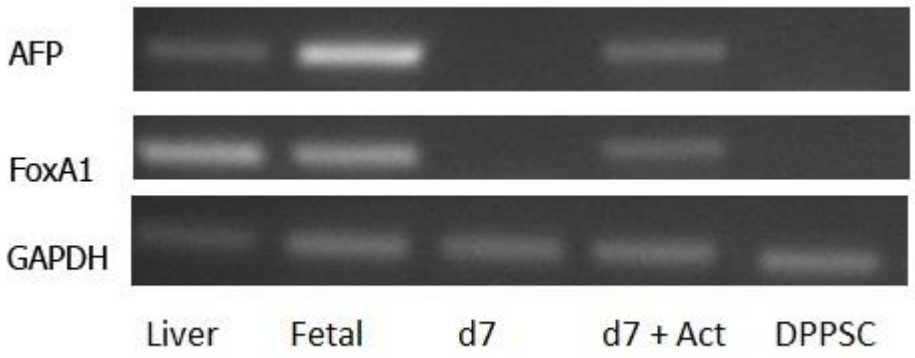


***Figure 33**. RT-PCR of early hepatic genes in DPPSC differentiated after 7 days with only 5 of them exposed to Activin A in comparison with cells exposed the 7 days to Activin A.*

Morphology of the cells was highly impacted during the differentiation. At the first stages, the cells were small and spindle-shaped. After the first days, the cells started to grow and they

became bigger in size and more elongated. After 2 weeks of differentiation the cells started to acquire an epithelioid morphology. They also stablished direct contacts with other cells of the neighborhood (Figure 34).

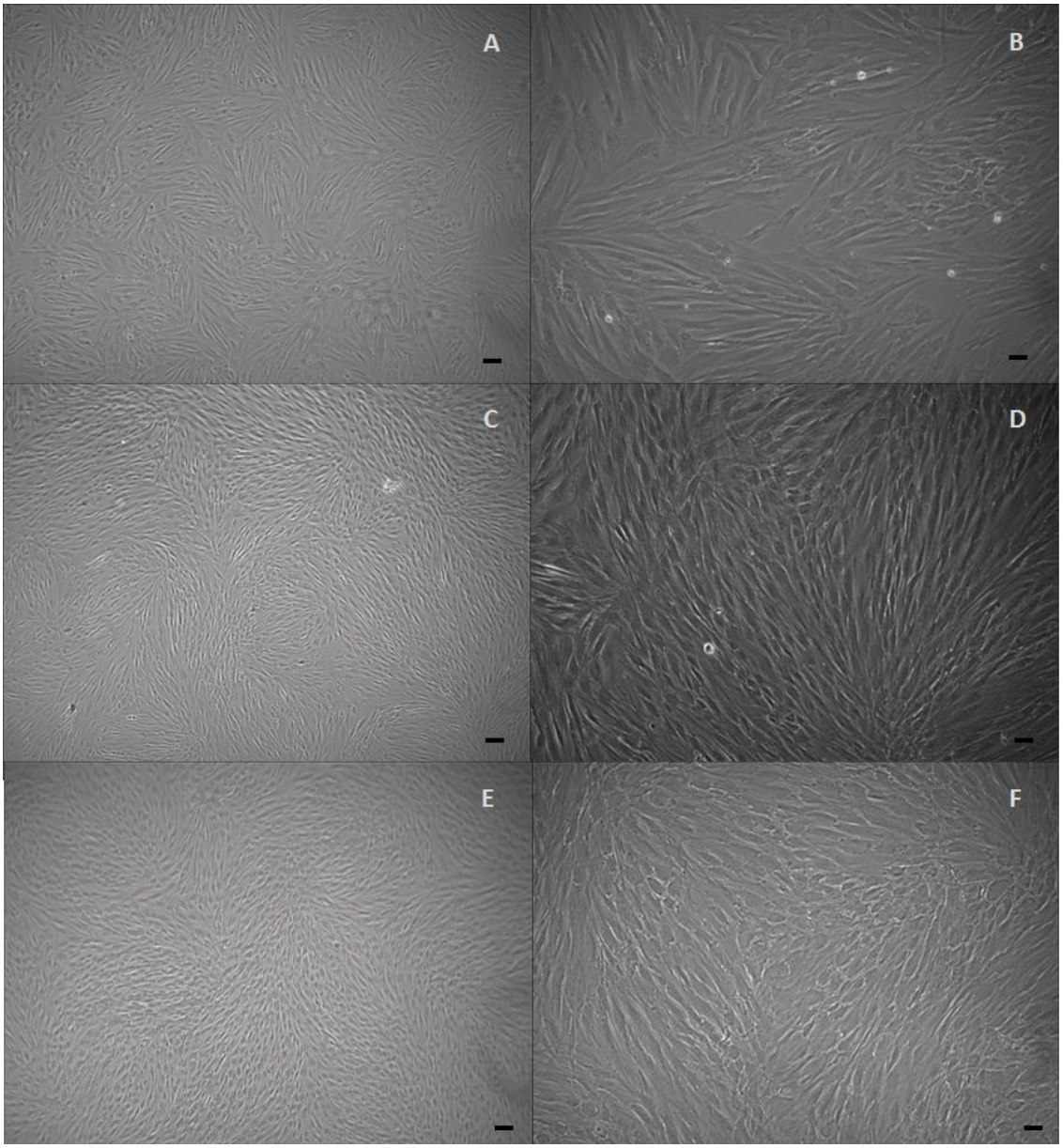


***Figure 34**. Morphology changes in differentiated DPPSC. **A-B**: Differentiated cells at day 7. **C-D**: Differentiated cells at day 15. **E-F**: Differentiated cells at day 22. Size bars: 100 µm (A, C, E) and 250 µm (B,D,F).*

We checked the expression of several hepatic-related genes throughout the differentiation process. The endoderm gene Foxa1 has its peak of expression after 7 days of differentiation and afterwards decreases to levels equivalent to undifferentiated cells with repeated increase at day 22. Regarding the mature hepatocyte genes AAT, G6P and ALB, we can evidence that they increase their expression throughout the differentiation with the highest expression at

day 22 of the differentiation. After 15 days, AAT and G6P were expressed at similarly high levels. However, the ALB gene needed the last week to get a substantial increase in expression. (Figure 35).

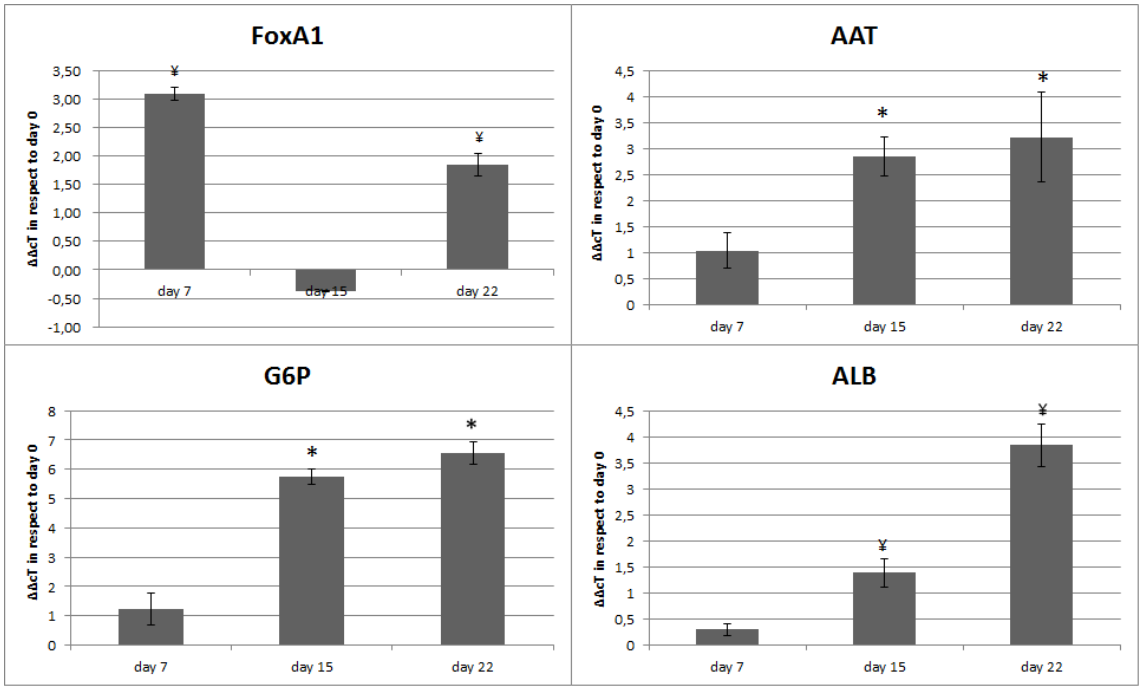


Figure 35. qRT-pCR of different genes throughout the differentiation process. Results are shown as ΔΔcT in respect to GAPDH and cells at day 0. *p<0.05 in respect to undifferentiated cells. ¥ p<0.05 in respect to all other samples (n=4).

Effect of a matrix overlay in the differentiation process

In several occasions, we have observed that during the differentiation process, there was a relevant amount of cell death, especially at late stages. We wanted to test if the contacts between the extracellular matrix and the cells could have an important role on such cell death so we tested if applying a matrix overlay on differentiated cells would be beneficial to the cells. The results show that cell death dramatically decreased upon culturing cells with the sandwich technology (Figure 36).

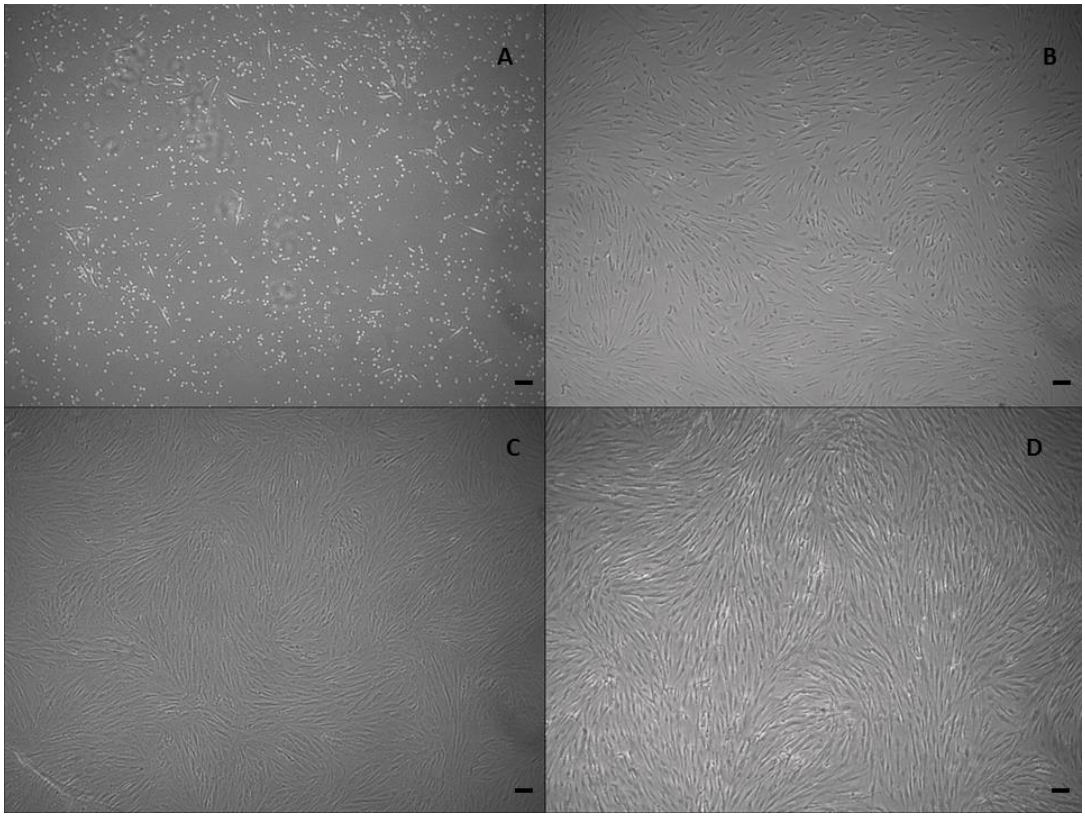


***Figure 36**. Changes in morphology of the differentiated cells after 18 days when they were untreated (A, B) or treated (C, D) with the Geltrex overlay. Size bars: 20 µm*

When checking the quantity of RNA of every sample in different the different conditions, the results confirmed what the morphology of cells had previously suggested to us (Figure 37).

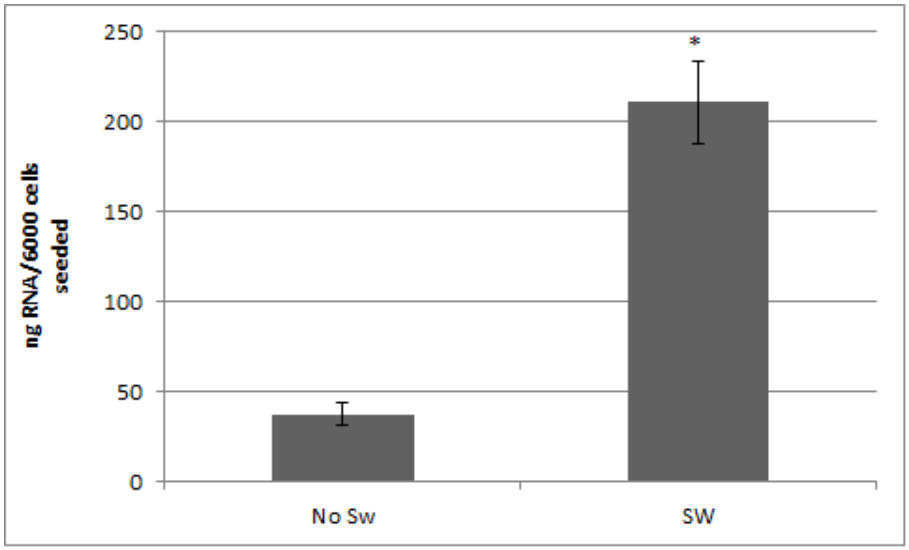


***Figure 37**. Cell death in differentiated cells with and without the Geltrex overlay. *p<0.05 (n=2)*

Furthermore, we wanted to know how it could affect the differentiation process aside of survival. Therefore, we evaluated the genetic expression of 2 different donors (#1 and #2) after 7 days (d7) of differentiation and after 14 days (d14). Also, in a few experiments, we analyzed the survival applying the matrix overlay over the cells at 7 days (sandwich, SW).

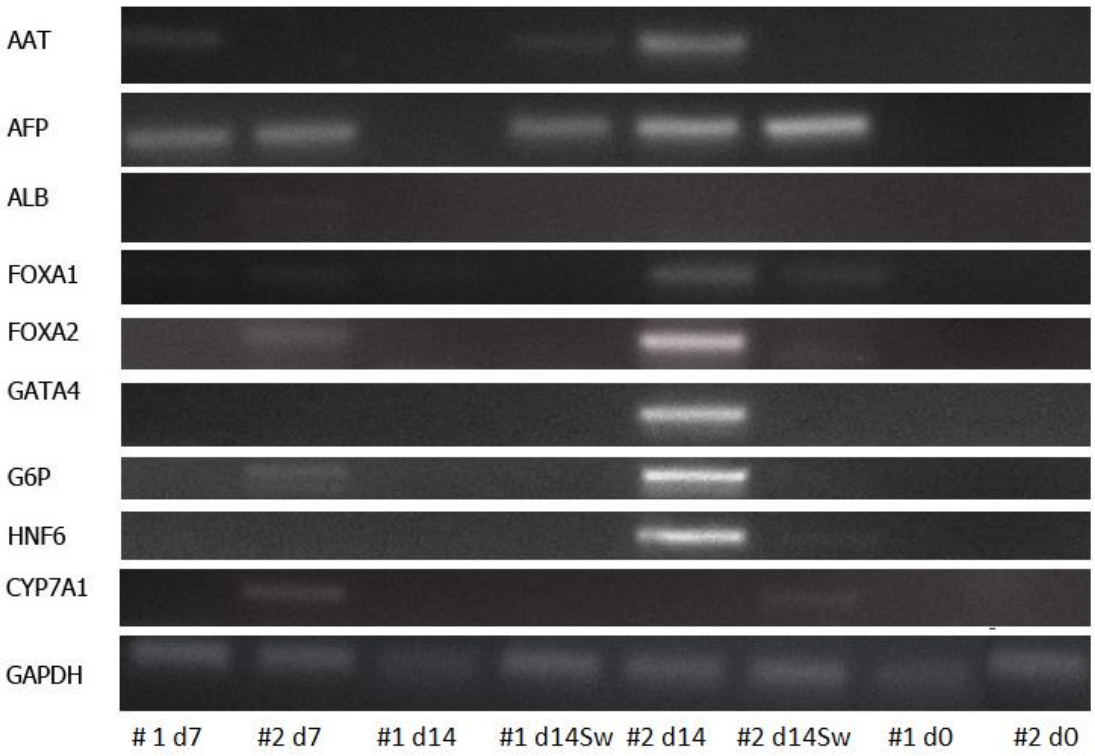


***Figure 38**. RT-PCR showing the effect of the sandwich technique in the expression of hepatic genes in DPPSC after 2 weeks of differentiation. GAPDH was used as loading control.*

The donor #1 only expressed AFP and a low amount of AAT after 7 days of differentiation, but after 14 days it lost the expression of such genes. However, the application of the sandwich technique allowed the maintenance of the expression of both genes. With the donor #2, the differentiation after 7 days was better as it showed expression of several hepatic-related genes (Figure 38). After 14 days, that expression was increased in most genes and it gained the expression of some genes such as HNF6 and Gata4. In the group of the sandwich, indeed, there was not a noticeable increase in the expression with respect to day 7 and it did not acquire the expression of the aforementioned genes that were expressed in the no-sw group. These results suggest that the geltrex overlay induces the cells to enter a stationary state where they cannot continue with their differentiation process successfully (Figure 38).

Then, we wanted to analyze if the time where the overlay was applied could have an influence on the efficacy of the differentiation. Hence, we applied the sandwich at 2 different time points (7 and 14 days) and checked the expression of several genes. We could observe that the expression of hepatic genes such as PxR, G6P and HNF6 was favored by the application of the sandwich overlay at day 7 and especially at day 14.

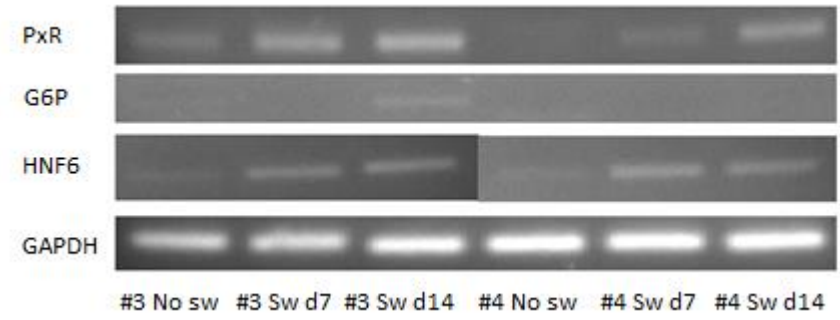


***Figure 39**. RT-PCR of PXR, G6P and HNF6 in DPPSC differentiated 22 days without and with sandwich applied at days 7 and 14.*

We wanted to confirm these results by qRT-PCR, so we checked the expression of AAT, ALB and G6P after 22 days both with and without matrix overlay with a large number of samples. In the case of G6P, there was an induction of expression in both samples after 3w in comparison with undifferentiated cells. However, the use of the sandwich culture was detrimental to the expression of this gene as it resulted in lower levels compared to the group cultured without the sandwich.

Albumin had similar results as G6P, after 22 days of differentiation both samples had a higher expression than undifferentiated cells. The use of the sandwich culture was again not efficient for the cell differentiation as they had lower levels of albumin and G6P than no-sw samples.

The AAT results were the ones that showed in a more clear way that the sandwich technique was not effective for the maturation of the cells. Only the no-sw groups had an increased AAT expression in relation to undifferentiated cells (Figure 40).

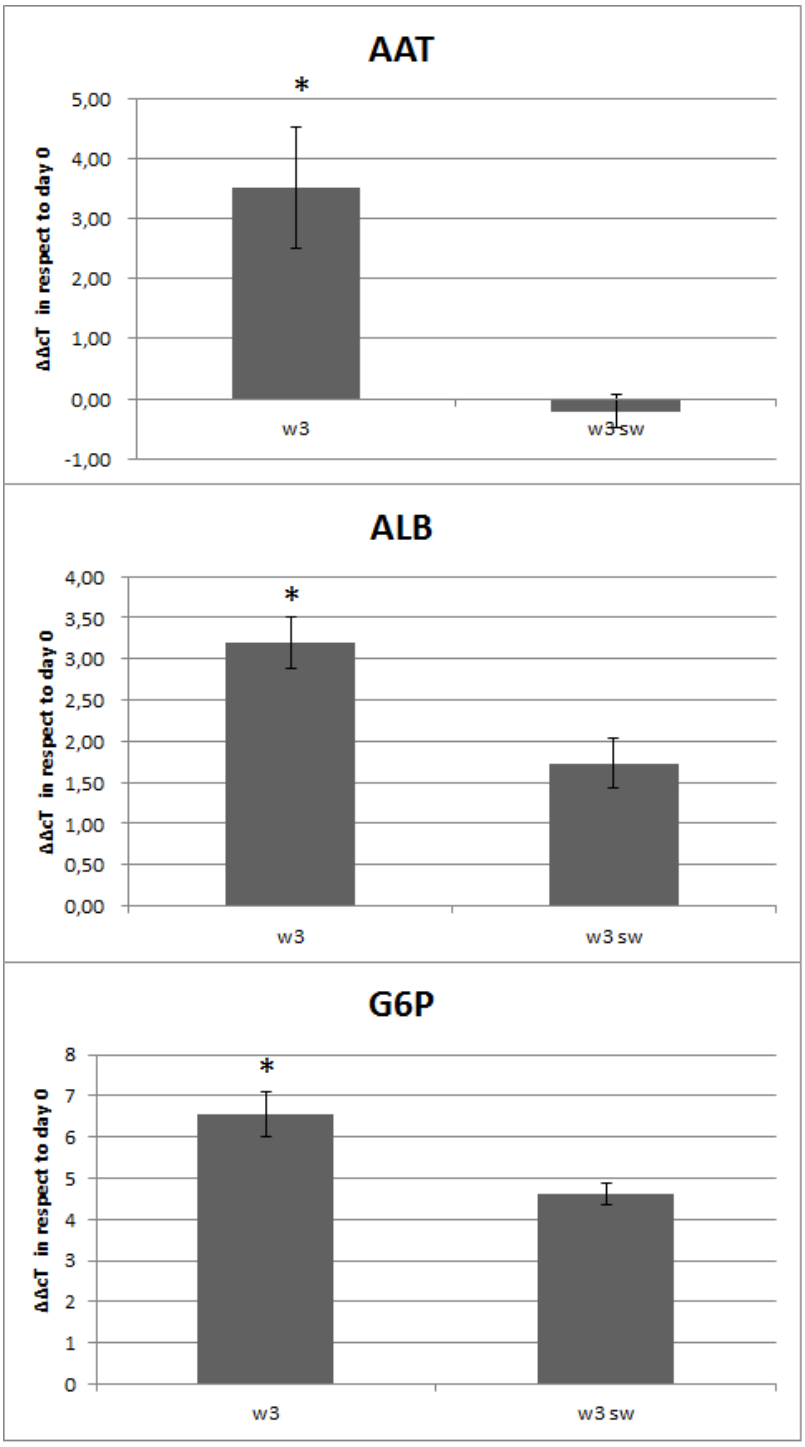


***Figure 40**. qRT-PCR of ALB, AAT, and G6P in DPPSC differentiated during 22 days with and without Geltrex overlay. Results are shown as ΔΔct in respect to GAPDH and undifferentiated cells. * represents p<0.05 in respect to the other samples (n=5).*

Protein and functional analysis of differentiated cells with the optimized protocol

After analizing the results obtained trying to optimize the generation of hepatocyte-like cells, we concluded that the best protocol for DPPSC consisted in 3 steps: first, using Activin for the first 7 days: secondly, FGF4 and HGF to specify hepatic fate (next 6 days of differentiation), and finally, HCM supplemented with FGF4, HGF, OSM and Dex for the last 9 days to induce maturation. We wanted to further characterize the cells obtained with this protocol so we proceeded to implement analysis that were not based on mRNA expression.

First of all we performed an immunofluorescence assay to see the expression of Ck18, ALB and AAT (Figure 41). Cells were positive to this three markers after 22 days of differentiation.

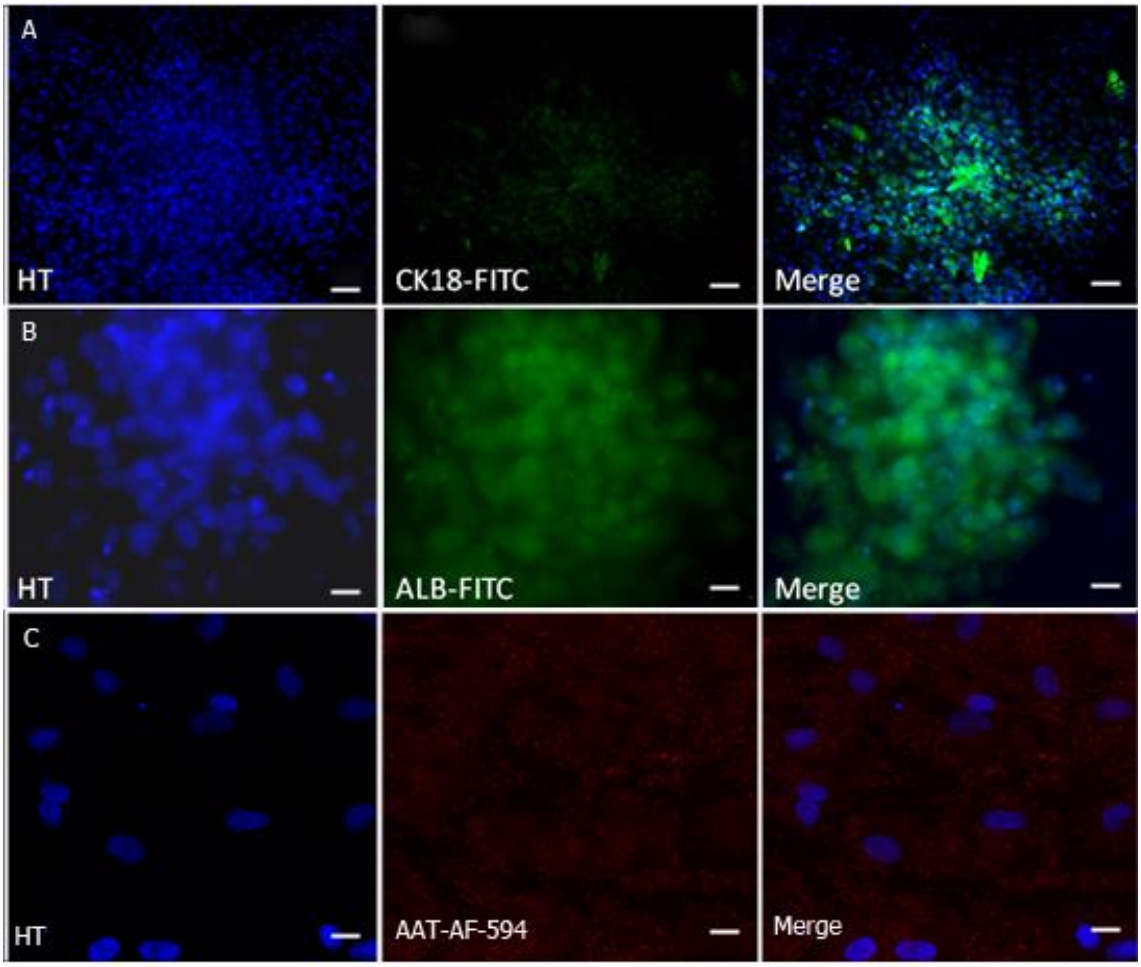


***Figure 41**. Immunofluorescence analysis of hepatocytes obtained from DPPSC. **A**: Staining with CK18. **B**: Staining with ALB. **C**: Staining with AAT. Size bars: 100 µm (A) and 10 µm(B, C).*

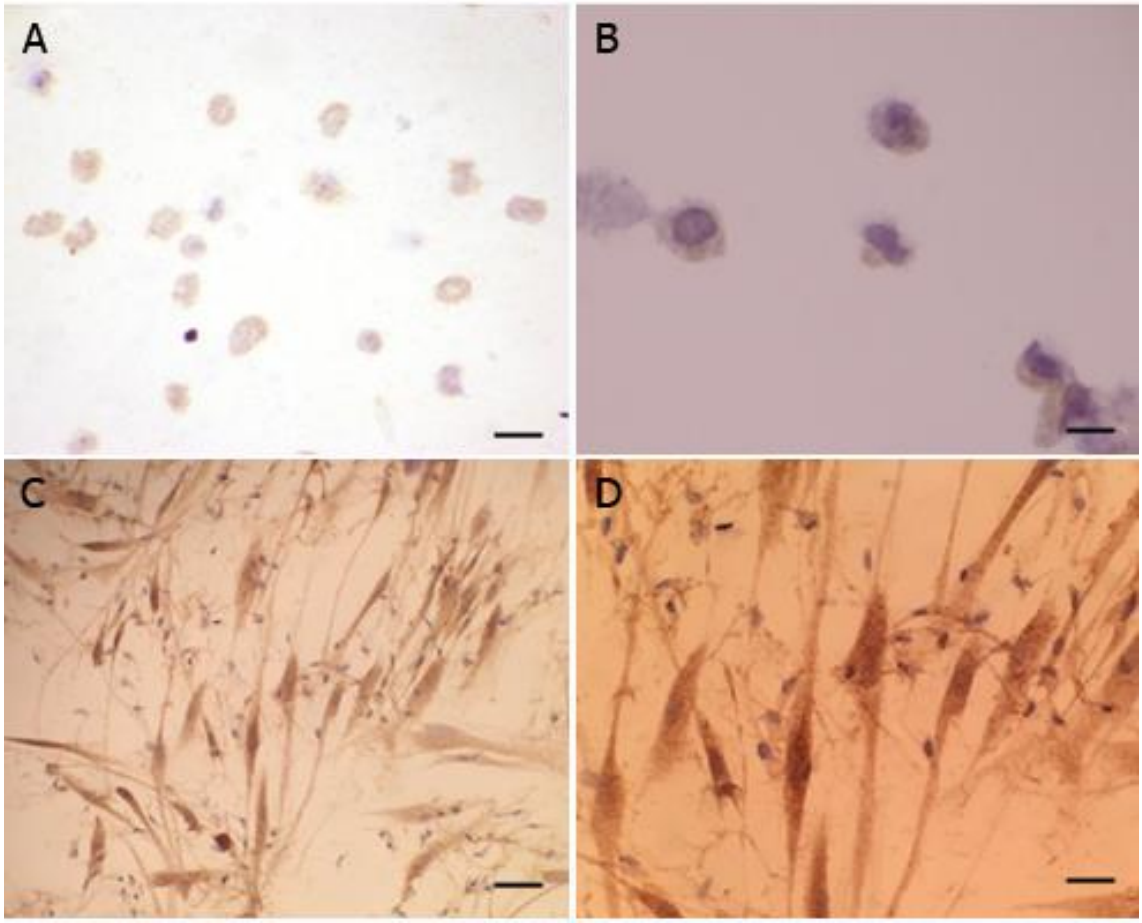


***Figure 42**. Immunocytochemistry analysis of DPPSC differentiated into hepatocyte-like cells. **A-B** Staining for AFP. **C-D**: Staining for AAT. Size bars: 40 µm(A,C) and 20 µm(B,D)*

We also analyzed the expression of hepatic proteins by means of immunocytochemistry and we saw that DPPSC differentiated into hepatocyte-like cells were positive for AFP and AAT (Figure 42).

In order to quantify the results of protein expression, we performed an analysis by flow cytometry showing the percentage of positive cells for a hepatic marker. In this sense, the flow cytometry results showed that 44% of the population was positive for AFP after 11 days of differentiation (Figure 43).

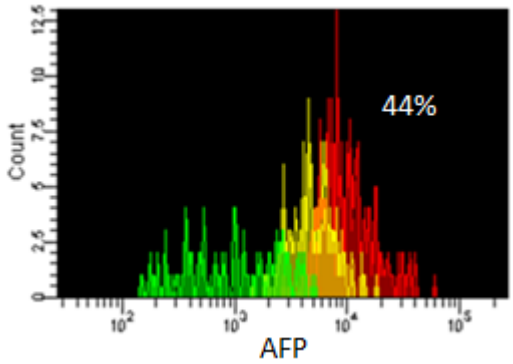


***Figure 43**. Flow cytometry showing the expression of AFP in DPPSC differentiated to hepatocytes after 11 days.*

We also wanted to check the ability of the differentiated DPPSC to perform diverse hepatic functions such as albumin secretion, glycogen storage and expression of cytochromes with activity. First, conditioned media were collected and it was measured their albumin concentration by a fluorometric assay. At early stages of the differentiation, the secretion of albumin was low. But from day 16, and mainly on days 19 and 22, the albumin secreted was significantly increasaed by the cells (Figure 44).

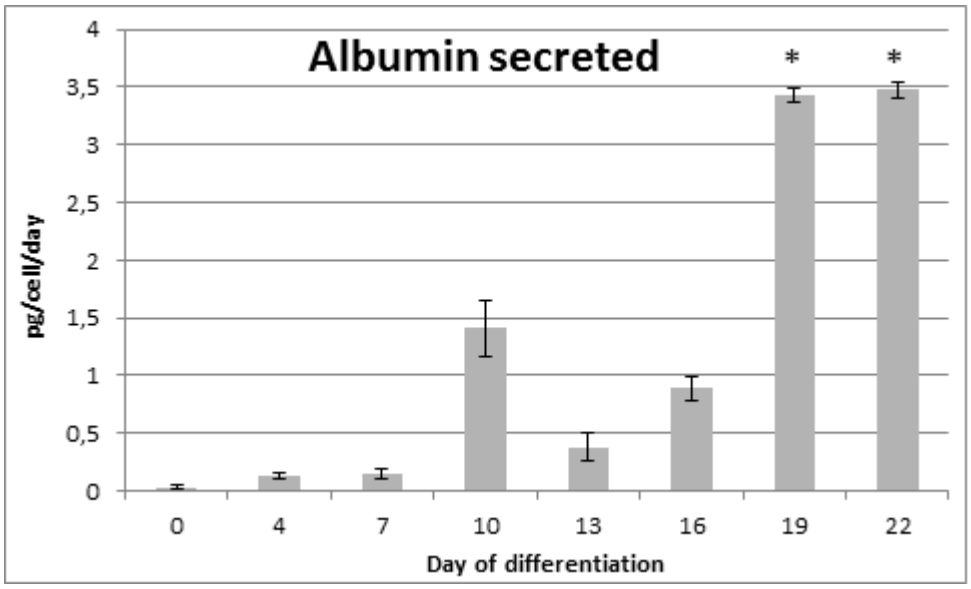


***Figure 44**. Secreted Albumin at different time points during differentiation of DPPSC. * p<0.05 in respect to days 0 to 16*

After the differentiation process, we checked the ability of the cells to store glycogen as it is an important function carried out by mature hepatocytes. The results showed PAS-positive staining of cells differentiated (Figure 45 D-F) in contrast to undifferentiated cells (Figure 45 A-C).

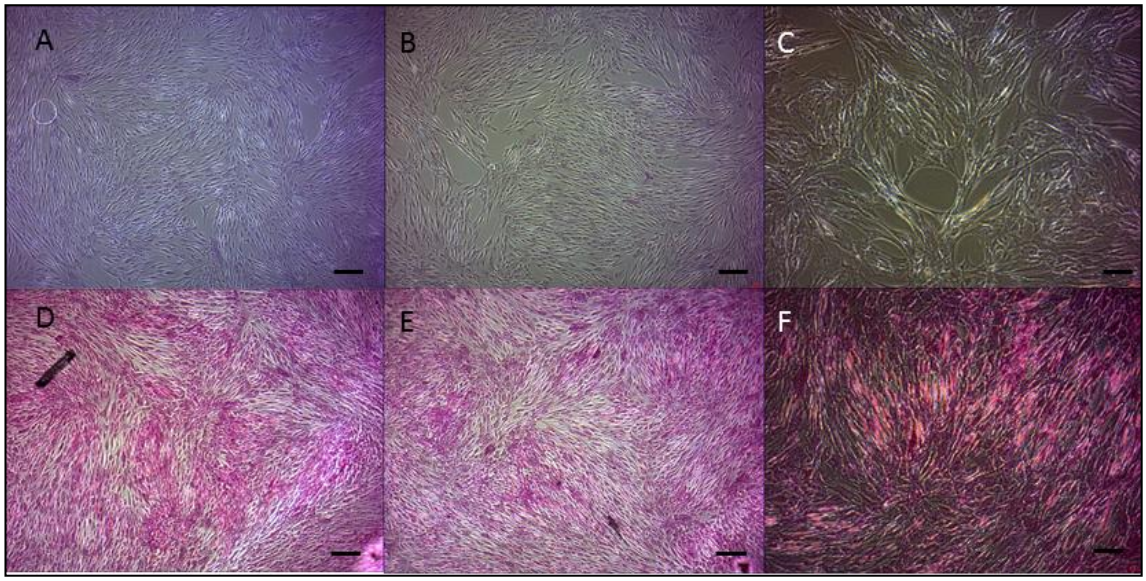


***Figure 45**. Cells stained with the PAS staining. **A**-**C** Undifferentiated cells **D-F**: Hepatic cells after 22 days of differentiation. Scale bar: 200 µM (A, B, D, E) and 80 µM (C,F)*

Another evidence of the maturation level of the hepatocyte-like cells generated from DPPSCs is related to the role of hepatocytes related to cytotoxicity and the metabolization of drugs through the cytochrome activity, especially from the CYP3A4 isoform. To check that, we measured the basal activity of the differentiated cells, their ability to be induced with a specific activator of CYP3A4 expression and also the inhibition of that activity. We used rifampicin as an activator of the cytochrome and erythromycin as a specific inhibitor. At day 22 of differentiation, the basal activity of the differentiated cells was only slightly higher than undifferentiated cells but they were able to increase their activity upon induction. Levels of activity were between 3 and 3.4 times higher after the 48 h treatment with rifampicin. The inhibition with erythromycin lowered the activity considerably, although not enough to get basal values. The CYP3A4 activity decreased between a 20 and 35% depending on the sample (Figure 46).

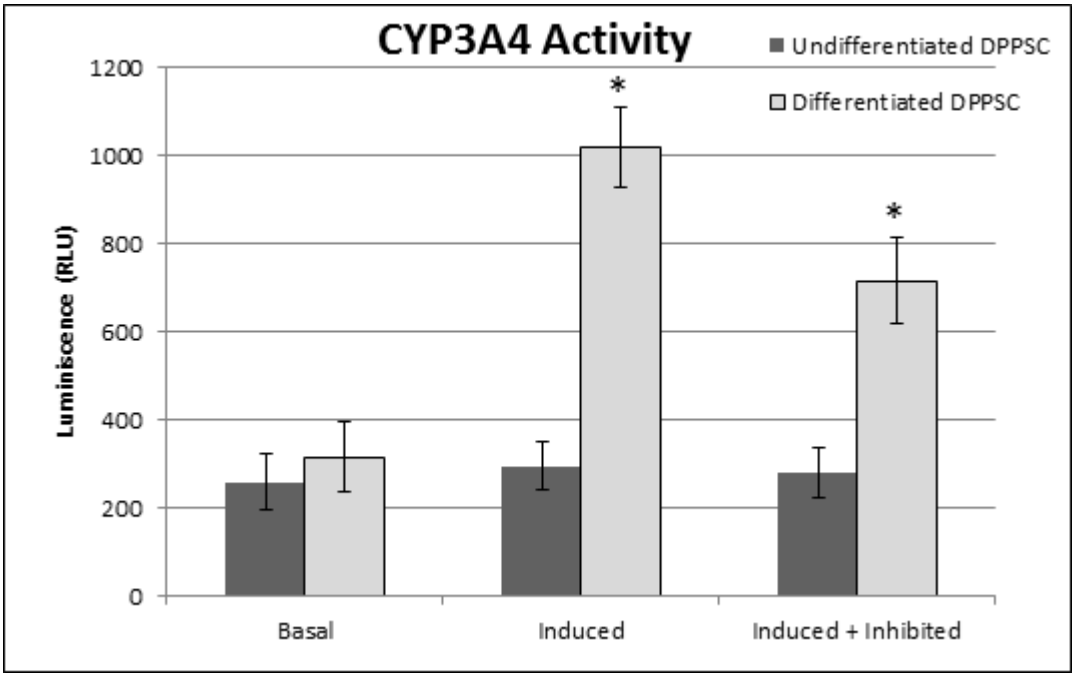


Figure 46. *CYP3A4 activity measured by luminescence in differentiated cells (after 22 days) and undifferentiated cells. * p<0.05 in respect to undifferentiated cells in the same condition*

DISCUSSION

Characterization of DPPSC

The results obtained in this study show that DPPSC are a source of stem cells with unique characteristics when compared to other adult stem cells regarding their expression of typical embryonic genes such as OCT4, NANOG and SOX2 that are known to be key in maintaining pluripotency (138).

Several stem cells populations have been isolated from different parts of the human tooth; however, all of them have been shown to have generic MSC-like properties (139). The first population isolated was the DPSC. They share several similarities with DPPSC as both of them are obtained from adult dental pulp. They share the expression of CD105, CD146, and lack the expression of CD45 and CD34. However, the main difference was the expression of Stro-1, showing a very low expression in DPPSC. We have demonstrated that DPSC and DPMSC can coexist in culture. Actually, MSC are known to be a very heterogeneous population in culture (140) hindering their isolation from the dental pulp and allowing the presence of DPPSC in cultures of DPMSC. However, we have shown that DPPSC are strictly dependent on their medium to maintain their phenotype so we can speculate that they would only coexist for a limited period of time and in a low number. Our experiments were focused on the detection of DPMSC presents in DPPSC cultures but it would be also interesting to test the other way around, which means the evaluation of the maintenance of a pluripotent-like stem cell population, like DPPSC, even in adverse conditions.

One of the main differences we have found in the protein expression is that, unlike MSC-like cells, DPPSC do not express CD73. As it has been shown previously with other pluripotent-like cells such as MAPC, the lack of CD73 can be a way to distinguish them from other populations from the same organ such as BMMSC (141). Some recent studies have demonstrated that MSC can influence other cells when cultured together regarding the expression of several membrane proteins, amongst them CD73 (142). However, in DPPSC there seems not to be such interaction, making CD73 a good candidate as a negative selection protein.

We have proven that DPPSC differ from DPSC in expression of embryonic markers such as OCT4, NANOG and SOX2. The low expression of Myc from DPPSC is a good sign, as it is a proto-oncogene that could lead to tumour formation, in possible future applications of DPPSC. Those genes were thought to be exclusive from pluripotent cells from embryonic origin, but in the last few years, several populations with such characteristics have been found in different human adult tissues. Most of them are found in the bone marrow: VSEL, MAPC (76, 143). Others can be found in other adult organs such as the heart or the liver (144). All of them have been demonstrated the expression of embryonic markers and a great potential of differentiation towards different germ layers. Other populations with no embryonic markers but with great plasticity have also been isolated from the BM and blood cord (75, 145). Regarding the dental pulp, there are also reports of populations expressing OCT4, SOX2 or NANOG when pulps were obtained from deciduous teeth. The cells have been called immature dental pulp stem cells (IDPSC) (146) or SHED. When they are obtained from the dental follicle, these cells are called DFC (147). Their relation with DPPSC remains unclear: they could have a common origin or be similar cells in different niches or found at different ages.

We have found that the pluripotency state in DPPSC is not definitive as culture conditions have an impact on it. In a similar way, Roobruck *et al.* (148) showed that MSC, MAPC and mesangioblasts (Mab) change their behaviour when cultured in other conditions, altering not only their expression protein pattern but also their subsequent ability to differentiate into different tissues. Fluctuation of pluripotency-related transcription factors has been previously documented in ESC and it has been proven that it does not disturb pluripotency. Usually cells with high expression of OCT4 have showed lower expression of other factors, such as NANOG and c-Myc and viceversa (149). Apparently, cells within a colony support each other in the maintenance of pluripotency. Previous studies have shown that small molecules such as rapamycin can affect dental pulp stem cell properties, especially regarding expression of OCT4, NANOG and SOX2 and also in their ability to differentiate (150). Our study was focused only in the changes in embryonic markers but it would be interesting to know how

changes in the culture conditions and small molecules could affect the differentiation abilities of both DPPSC and DPSC.

Moreover, knowing further information about the kinetics of these changes would be interesting. For instance, we only checked the expression of pluripotent stem cell markers at one single timepoint but it will be interesting to evaluate them at different timepoints in order to determine the exact timepoint when the change took place. Knowing if that tendency could be kept for a longer period of time or whether the cells would be able to revert to their initial state if conditions were changed back to normal would clarify some of the concerns regarding this ability. Clonal studies would be very useful in this regard, but the susceptibility of the DPPSC to excessive manipulation and cell sorting have been hurdles unavoidable until now.

In that regard, it is interesting to discern what the role is of each of the three main growth factors that are used in the culture medium of DPPSC: LIF, EGF and PDGF. Concerning EGF and PDGF, they have been shown to promote proliferation and migration of BMMSC (151). Their role in the differentiation capacities is more controversial: some studies report that EGF enhances osteogenic differentiation of MSC (152) while others claim that EGF alone does not enhance either osteogenic abilities or adipogenic/chondrogenic lineages. Apparently concentrations and exposition to EGF make a difference, as low temporary stimulation by EGF seems to have anti-osteogenic effects while strong and sustained stimulation favours the osteogenesis (151).

Other studies have demonstrated an important role of PDGF in maintaining hESC pluripotency (153), although it was not the only component required in the media components. Other studies have shown its anti-apoptotic effect on hESC (154).

The role of LIF in human cells is very different compared to other animal models' stem cells as it is required for the maintenance of mESC but not for hESC (155). However, in the last years, different states of pluripotency have been discovered in hESC called primed and naïve, being the latter dependant on LIF/Stat3 signalling (156). The role of LIF function in ASC has not been extensively studied, but there are studies that show

that it can strengthen the expression of OCT4, NANOG and SOX2 in ADSC (157) as well as enhance the expansion of germinal stem cells in feeder-free conditions (158).

Cell density is also known to affect cell behaviour, especially regarding expression of embryonic genes. BMMSC have been shown to express higher levels of OCT4 and NANOG when cultured at low densities (200 cells/cm^2) in comparison with cultures at higher densities (1000 and 5000 cells/cm^2) (159).

With all this information in mind, it is assumable that the DPPSC culture condition must have a direct contribution on the particular genetic expression of DPPSC.

One of the methods used to evaluate pluripotency is the ability of cells to form EB, an structure characterized by containing cells of the three germ layers (80). We have demonstrated that DPPSC form EB and that these EB enhance the expression of specific genes from the three embryonic layers. However, cells that form EB are supposed to lose their embryonic characteristics to be able differentiate to different tissues, so one could envision that EB from DPPSC are not fully developed EB as they retain the expression of embryonic markers. Nevertheless, there is some evidence with mESC showing that after several days in the EB the cells start recovering the expression of OCT4 and maintain it for more than 2 months (160). Other studies show that DPPSC are not the only post-natal population able to develop EB, as cells from the neural crest can also form sphere clusters with similar characteristics from those of DPPSC's EB (eg. expression of pluripotency markers) (147).

The other known method to assess pluripotency is the teratoma formation. We have demonstrated that DPPSC were able to lead the formation of a teratoma-like structure when they were injected subcutaneously in immunodeficient mice that included chondrogenic tissue, adipogenic tissue, osteogenic tissue, vascularized tissue, and duct-like structures. This result is a drawback in future applications of DPPSC *in vivo*. The results were negative when DPMSC were transplanted in the same mouse strain, being another piece of evidence that proves the different behaviour between both populations and the higher differentiation potential that DPPSC own. The presence of embryonic markers after culturing the DPPSC for several weeks suggest that cells did

not reach their more differentiated state at that point, so further experiments with longer periods of formation may be needed.

The relationship between DPPSCs and iPSC should also be investigated. The induction of iPSC seems to be easier from cells that already have high levels of expression of certain factors such as SOX2 or c-Myc (161). We can speculate that this could be the case of DPPSC as they have high endogenous expression of OCT4, NANOG and SOX2 and they could be potentially reprogrammed with only 2 factors. Further studies are needed to answer these questions.

For therapeutic purposes, the reliability and safety of putative clinical applications for DPPSCs must be considered, especially the issue of genetic stability. We have demonstrated that DPPSCs show no chromosome abnormalities when cultured *in vitro*, such that we propose that DPPSCs are safe to use for clinical therapies. We propose that short-CGH be used in stem cell research to determine genetic stability when cells are cultured *in vitro*, because s-CGH allows the detection of genetic abnormalities that could remain hidden with the current protocols, such as karyotype or FISH techniques (137). In the same direction, it needs to be considered future changes in the culture conditions in order to establish a GMP protocol that allows the isolation and expansion of DPPSC with defined culture conditions. This has been already done with hESC (162) and other populations of ASC (163), meaning that it could feasible in the case of DPPSC.

Obtaining DE

The results shown in this work indicate that DPPSC are a good candidate to generate hepatocyte-like cells by using a protocol that resembles liver development. There are several protocols published proven to be successful in generation of hepatocytes from stem cells. However, there is a lack of standardization on the evaluation step of the differentiated cells derived.

The use of EB as a first step of differentiation was the most common a few years ago, but several publications have demonstrated that it was not always good enough for the differentiation due to the low efficiency of the process, high heterogeneity and

disproportional exposure to exogenous factors that limited the expansion and differentiation of the cells (86). In the case of DPPSC, we have proven that they can form EB by several methods and that these EB had upregulated the expression of some endoderm markers. Unfortunately, it was not an efficient method for the further differentiation of DPPSC into hepatic cells.

Regarding the differentiation protocol, Activin A is the main factor needed to induce definitive endoderm *in vitro* (83, 84). It is well known that Nodal signaling is required for endoderm specification during the gastrulation and Activin A is the only source for such activity *in vitro* (82). Our results confirm that Activin A is essential to induce endoderm specification in the differentiation with DPPSC. DE induction seems to be dependent on the time cells are exposed to the factors. Some studies have found a peak on DE after 5 days of induction, but others have proven that 7 days is a better time to differentiate towards DE and cells had higher expression of genes such as Alb at the end of the differentiation (164). In this regard, we have confirmed that 7 days of exposure to Activin A is the optimal time to obtain the higher percentage of cells positive for endoderm genes.

BMPs have been shown to have a role in the development of the liver, but their efficacy regarding *in vitro* differentiation from adult stem cells remains unclear. Some results have proven that cells do not increase the expression of hepatic markers under the influence of BMP2 or BMP4 (165) but the contrary. Others authors have proved that endoderm induction by Activin A is not adversely affected by the addition of BMP4 in hESC. In this direction, our results confirm that BMP4 does not affect negatively the endoderm induction by Activin A in DPPSC but it is not beneficial either when compared to Activin A alone. Some studies have suggested that Activin A combined with BMP4 induce mesoderm formation and is useful for the production of cardiomyocytes (166).

The combination of Activin A and Wnt3a is widely used since it has been shown to be effective to induce endoderm (83, 164). Some studies have shown that it can be considered better than the induction from Activin A alone or in combination with BMP4 (167). In the same way, our results suggest that Wnt3a in combination with

Activin A was better than other growth factors such as FGF4 or bFGF for endoderm induction. However, in our hands, Wnt3a did not have a significant impact and only one gene (Foxa1) was upregulated in comparison with Activin alone.

Recently, some researchers have started to address the activation of the Wnt pathway by directly inhibiting the signaling molecule GSK-3 with specific inhibitors such as CHIR99021 or 6-bromoindirubin-3'-oxime resulting in a higher induction of endoderm (168). Fortunately the price of such inhibitors is lower than the Wnt3a molecule, so it opens new pathways to optimize protocols in endoderm and hepatic differentiation.

Some studies have reported that the combination of Wnt3a and BMP4 without Activin A is enough to induce the definitive endoderm in hESC (169), however we did not consider this option in our experimental design. Another combination of factors also unexplored by us was Activin A together with Wnt3a and HGF for endoderm induction. There is some evidence that the three cytokines act synergistically to induce endoderm in hIPSC, accelerating the expression of genes such as Sox17 and Foxa2 (164). However, the lack of more studies supporting this information made us discard it as an option to test its effects on the differentiation from DPPSC.

The concentration of FBS used in the induction of definitive endoderm seems to have a critical role as inhibition of PI3K would be necessary to allow the effects of Activin A. Some reports suggest that a concentration of 0,5% during the first 5 days is the optimal amount of FBS to enhance the expression of genes such as Sox17 or Foxa2 in hESC (83). In our protocol, the first 3 days of differentiation included 0.5% FBS but afterwards it was changed to 2% KOSR as it has been proven to be equally effective and is more suitable for being a defined component (89). In our attempts to remove completely the FBS from the protocol, we tried to replace it with KOSR for the first three days of differentiation. However, the results were not positive as the expression of Foxa2 decreased in comparison with cells treated with FBS for the first days. Therefore we concluded that DPPSC needed these first days in contact with FBS in order to differentiate properly into DE.

Hepatic specification and maturation

Our protocol uses FGF4 and HGF as hepatic inductors and OSM with Dex for the hepatic maturation. Many authors have demonstrated that those factors are determining in hepatic differentiation because they are known to have an important role in liver development and they have also been effective *in vitro* for maintaining hepatocytes cultured or to differentiate stem cells.

It has been documented the use of FGF4 and HGF as inductors of hepatic differentiation in different studies with cells from different origin (119, 134). HGF is a key factor for liver growth and function (170). In differentiation protocols from stem cells it has been suggested to have an important role in the hepatic specification and induction of ALB^+ cells. Its role in obtaining definitive endoderm has also been proven in iPSC (171). FGF4 is a mitotic agent for fibroblasts and endothelial cells. Both have an important role in liver development, but it is unclear if they can induce definitive endoderm without the contribution of other factors. Our results with DPPSC suggest that they need the assistance of other factors in order to induce a fully hepatic fate. When we combine HGF and FGF4 alone, the expression of some hepatic genes could be seen, but not in a reproducible way. Some studies have suggested that there is a need to expose cells to FGF4 and HGF in a sequential way in order to improve the differentiation efficacy (172). In our final protocol we used them to induce hepatic fate after 7 days of differentiation and probably the first steps of induction to definitive endoderm would not be fulfilled with their only effect. After 14 days of differentiation, cells have acquired a high level of expression of several hepatic markers including immature and mature genes, so the role of FGF4 and HGF is demonstrated as it is after the exposure to them when cells acquire this phenotype.

Other members of the FGF family have been tested and it seems that FGF2 and FGF1 could have an impact on definitive endoderm induction and hepatic differentiation in hESC (167). It could be interesting to test how they could affect the differentiation from DPPSC. Our experiments with bFGF confirm the results previously shown by Schwartz *et al.* (173) as that factor did not induce endoderm formation in DPPSC.

OSM is an interleukine-6 family cytokine and it is required for maturation of hepatocytes both *in vitro* and *in vivo* in combination with glucocorticoids such as Dex (174, 175). It is known that HGF, EGF and OSM have decisive effects on the maintenance of primary human hepatocytes *in vitro* (176). The combination of HGF, OSM and Dex is widely used in protocols to differentiate cells to hepatocytes (89, 177).

In our protocol, the application of the maturation factors OSM and Dex occurs at day 13 when the cells already are committed to the hepatic fate. Our results show that all the features of mature hepatocytes upregulate after cells have been exposed to OSM and Dex for several days. The changes in morphology are more evident after 15 days, and the production of Albumin and enzymes as GGT and AST peaks after 18 days, confirming that the maturation from DPPSC to hepatic cells relies heavily on these factors.

Culture support

Lately, differentiation protocols have incorporated different culture systems beyond the traditional two dimensions culture system that better emulates the real situation during liver development. The use of three dimension conditions to grow the cells improves the efficiency of the differentiation as 3D culture systems more closely resemble the *in vivo* environment in regards to inducing correct cell morphology, cellular environment, gene expression and biological behaviour of the cells (81, 90). Our results, however, show the opposite. Although the 3D glass scaffold has been proven to be effective in DPPSC differentiated to osteoblasts (79), probably the characteristics of the material are not adequate for hepatocytes where the extracellular matrix has a much bigger impact. Usually, natural hydrogels such as matrigel and alginate are used due to their biocompatibility, mild gelling conditions, and good cell entrapment properties; they mimic *in vivo* architecture and provide easy manipulation of cells and tissues as well as promoting cell-matrix interactions. The major disadvantages of using natural materials are the limited control over physiochemical properties that they offer, as well as immunogenicity, degradability, lack of reproducibility, and inconsistency in mechanical properties (178). The advantage of using synthetic polymers is the wide choice they offer in design

parameters including porosity, pore connectivity, pore geometry, pore size distribution, and surface topography.

The ECM is comprised by four macromolecular groups: collagen, elastin, structural glycoproteins and proteoglycans. ECM is the modulator of numerous biological processes by its capacity to deliver solid- phase signals through selective binding and subsequent release of growth regulating factors and its interaction with cell surface receptors. Several studies have indicated a critical role for the hepatic ECM in the liver homeostasis. Extracellular matrix isolated from liver tissue has been shown to maintain the phenotype of hepatocytes in culture. It has also been shown that optimization of the physical and chemical properties of ECM is important for the maintenance of hepatocyte function *in vitro* (178).

Regarding extracellular matrices, some reports show that Matrigel can induce a higher cellular proliferation and longer survival of hepatocyte-like cells (165). However, other studies that have compared Matrigel, fibronectin and collagen I , showed that the collagen support efficiently the differentiation of hESC into hepatocytes (173). Our results show that both collagen and fibronectin offer good conditions for DPPSC to develop an endoderm phenotype so we continued with the use of Collagen I as it is the most widely used ECM protein in hepatic differentiation. Results with Matrigel are more prone to be variable and reduce reproducibility so we preferred to avoid it after proving other matrices were good enough.

Sandwich culture has been very useful over the past years to improve the culture of hepatocytes *in vitro*. This model facilitates the preservation of certain liver characteristics including cuboidal morphology with features such as bile canaliculi, tight junctions and gap junctions. In addition, appears to enhance expression of liver-selective proteins such as albumin and contributes to decreased levels of spontaneous apoptosis and oxidative stress (179, 180). However, the use of the sandwich culture has been barely used in differentiation protocols. Some of the existing studies show that a Matrigel overlay enhances the expression of endodermal genes and proteins in hESC and decreases the expression of genes related to mesoderm (181).

In our protocol, we did not face adversities when inducing the endoderm but at later stages of the differentiation where there was a high number of dead cell. For this reason, we have applied the overlay at day 7 and 14 of the differentiation. Our results showed that the overlay was not beneficial for the complete maturation of the differentiated cells since the sandwich technique favored a stationary state of differentiation but did not to enhance it or accelerated it.

Some recent reports use both the co-culture and the sandwich technique to improve the differentiation process in iPSC (182). The results are promising so it can be a good alternative to consider in the differentiation with DPPSC.

Co-culture

Heterotypic interactions play a fundamental role in liver function: they mediate in the formation of this organ from the endodermal foregut and mesenchymal vascular structures and they are also implicated in adult liver physiology. Hepatocyte viability and liver-specific function have been shown to be stabilized for several weeks *in vitro* upon co-cultivation with a variety of other cell types (183).

We wanted to evaluate if a co-culture with HepG2 cells could be beneficial for obtaining definitive endoderm from DPPSC. The results showed that cells acquired a fibroblast-like morphology and proliferated massively. Probably HepG2 secreted cytokines that favored proliferation rather than differentiation; consequently the generation of endoderm process was not improved when the co-culture with HepG2 was applied. As HepG2 resemble a more mature hepatic phenotype, their use at later stages of differentiation could have been more useful, as it has been shown in some experiments with other hepatic cell lines (184). However, the aim of this experiment was to improve the production of definitive endoderm so we discarded the co-culture as a method of support for our differentiation. The use of endodermal cell lines or applications of the co-culture at different time points could be other paths to explore in the future.

Evaluation of the differentiation

Several studies have been published demonstrating that stem/progenitor cells can be differentiated towards ‘‘hepatocyte-like cells’’ However, the criteria to identify a differentiated cell as a hepatocyte have not been standardized and differ greatly among different studies. Nonetheless, there are some common assays that are widely used to characterize the cells obtained after the differentiation. These include: evaluation of the morphology and ultrastructures of the cells, checking the genetic expression by quantitative methods, checking the protein expression, testing the functional abilities of the hepatocyte-like cells and finally evaluating their behavior *in vivo* (185).

After the establishment of the most optimal protocol used for DPPSC, we performed all the tests that were available for us in order to prove the efficacy of the differentiation of DPPSC into hepatocyte-like cells. Regarding genetic expression, differentiated DPPSC were able to express genes such as AAT, ALB and G6P that are typical of mature hepatocytes, as well as other endoderm-related or immature hepatic genes during the differentiation process (eg. Foxa2, Foxa1, Gata4 and AFP). However, there are many other genes that characterize hepatocytes so it will probably need to prove the expression of them as well. The protein expression has also been checked successfully for several proteins: for the early stage of differentiation AFP and Ck18 and for later stages ALB and AAT were detected.

Changes in morphology throughout the differentiation are very clear, acquiring the polygonal shape typical of hepatocytes and stablishing cell-cell contacts that are known to maintain hepatocyte polarization and functionality (5).

Aside from that, it seems reasonable to introduce additional criteria to define if a cell is a true hepatocyte or only shares several characteristics with a hepatocyte (185). Hepatocytes are cells that are able to perform a wide range of functions including glycogen storage, secretion of albumin, secretion of several metabolic enzymes and detoxification procedures, among others. The levels of albumin secreted in cells differentiated from DPPSC are at least as good as those found in other publications

from other stem cells (186, 187). The ability to accumulate glycogen, although hard to quantify, seems to be very high on differentiated DPPSC.

Probably the most uncertain aspect regarding hepatocyte functionality is the drug metabolism, a field where hepatocyte-like cells would be very useful for the pharmaceutical industry. However, most reports of cells differentiated to hepatocytes have shown activities far from those of primary hepatocytes (93). In the case of differentiated DPPSC, we have shown a high activity of CYP3A4 and high ability to be induced. All these results lead us to think of DPPSC as a promising source of functional hepatocyte-like cells. However, for clinical applications there are still many studies that need to be done. *In vivo* approaches to check the behavior of the hepatocyte-like cells within a live organism will be crucial to determine their applicability in therapy.

Despite the promising results, the differentiation status of hepatocyte-like cells from adult sources has not been characterized as well as from the pluripotent stem cells. The percentage of hepatic lineage cells tends to be different when different markers are used and the nature of the rest of the population is usually not reported. So far the efficiency of hepatic differentiation from adult tissues is still insufficient for clinical application in bioartificial liver or cell transplantation, which might be improved by modifying culture conditions or adding various growth factors/cytokines.

For liver repopulation, however, there are some studies that suggest that mature adult hepatocytes are the better candidates whilst other studies suggest that fetal liver cells might be superior as they have a greater proliferation potential and exhibit less apoptosis following transplantation (185).

The results shown in the present dissertation lead us to think of DPPSC as a promising source of functional hepatocyte-like cells. However, for clinical applications there are still many studies that need to be done. *In vivo* approaches to check the behavior of the hepatocyte-like cells within a live organism will be crucial to determine their applicability in human cell therapy clinical trials.

CONCLUSIONS

1. DPPSC have different genetic expression than DPMSC: they have a higher expression in embryonic genes such as OCT4, NANOG, SOX2 and a lower expression of CD73. Although they can coexist in culture for a period of time, these markers can be used to distinguish both populations.
2. The phenotype of DPPSC is influenced by the culture conditions and it can change when the conditions change. DPPSC show no chromosome abnormalities when cultured and expanded *in vitro*.
3. DPPSC can develop embryoid bodies that have upregulated the expression of genes representative of the 3 germ layers and they can form teratoma-like structures when injected into immunodeficient mice that include chondrogenic, adipogenic, osteogenic, vascular, and duct-like tissues.
4. Activin A alone, or combined with either Wnt3a or BMP4 are efficient ways to induce definitive endoderm in DPPSC. Collagen I and FN are suitable matrices to support endoderm differentiation in DPPSC. FBS is required for 3 days in DPPSC to induce definitive endoderm.
5. DPPSC are able to generate hepatocyte-like cells that resemble hepatocyte morphology, express several hepatic markers at the levels of mRNA and protein and that are able to carry out functions typical of hepatocytes.
6. A matrix overlay has an impact on the hepatic differentiation of DPPSC but does not enhance it.

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APPENDIX

Resumen

Introducción

El hígado es el órgano interno más grande del cuerpo humano y desarrolla funciones vitales en cuanto a metabolismo. El 70% de la masa de un hígado adulto lo constituyen los hepatocitos, que son células parenquimales que, junto con los colangiocitos, derivan del endodermo embrionario.

Los hepatocitos producen la mayoría de proteínas circulantes en sangre, incluyendo transportadores (albúmina, ceruloplasmina, transferrina, lipoproteínas), inhibidores de proteasas (α1-antitripsina, antitrombina, α2-macroglobulina), factores de coagulación (fibrinógeno, protrombina, factores V, VII, IX, X de coagulación) y moduladores de complejos inmunológicos e inflamación (complemento C3, proteína C reactiva). También controlan la homeostasis de glucosa/glicógeno y de los ácidos grasos como triglicéridos, colesterol, ácidos biliares y vitaminas A y D. Los hepatocitos metabolizan aminoácidos, metales (como el cobre y el hierro) y componentes endógenos como el grupo hemo y la bilirubin. Además, tienen un papel crucial en la detoxificación de xenobióticos y medicamentos.

Debido al gran número de funciones desarrolladas por el hígado, las enfermedades hepáticas resultan en grandes índices de morbidez y mortalidad, siendo, por ejemplo, la cuarta causa de muerte entre personas de mediana edad en los Estados Unidos. Algunos de los factores de riesgo relacionados con las enfermedades hepáticas son la obesidad o exceso de peso, alcoholismo y reiterada exposición a ciertas toxinas y medicamentos.

Un ejemplo de enfermedad hepática es la cirrosis, que es responsable del 1,8% de muertes en Europa (170.000 cada año). La cirrosis puede a su vez desencadenar en hepatocarcinoma, la forma más común de cáncer de hígado, que es responsable de 46.801 muertes en Europa al año. La hepatitis es también una de las causas importantes de enfermedad hepática, siendo las más extendidas las causadas por los virus de la hepatitis A, B y, sobre todo, C.

Todo esto son ejemplos de algunas de las enfermedades que afectan al hígado y que, en la mayoría de ocasiones, pueden ocasionar fallo hepático. El único tratamiento actual para las fases avanzadas de estas enfermedades es el trasplante de hígado, que actualmente es un tratamiento rutinario con una tasa de supervivencia después de 5 años de más del 70%. Más de 5.500 trasplantes de hígado se realizan en Europa cada año. El mayor inconveniente que afronta el trasplante de hígado es la creciente discrepancia entre el número de personas que lo necesitan respecto al número de donantes, lo que ocasiona la muerte de aproximadamente el 15% de los pacientes en espera de un trasplante de hígado.

Debido a esta escasez de hígados disponibles para ser trasplantados, el trasplante de hepatocitos aislados se está viendo como una alternativa prometedora para varias aplicaciones clínicas. Los hepatocitos se extraen de hígados que no son aptos para los trasplantes, incluyendo hígados de donantes por muerte cardíaca. Una de las ventajas de este procedimiento es que los hepatocitos se pueden usar inmediatamente o se pueden congelar para su uso en tratamientos de emergencia, aunque la congelación reduce su viabilidad. Sin embargo, no todo son ventajas con el trasplante de hepatocitos: hay poca eficiencia de

implantación, muchas células no sobreviven largos periodos después de entrar en el hígado o pierden su función, mientras que otras se distribuyen ectópicamente. Además, la disponibilidad de hepatocitos, aunque mayor que la de hígados enteros, sigue sin ser suficiente para la gran demanda.

La mayor aplicación para el trasplante de hepatocitos son las enfermedades metabólicas hereditarias, en las cuales se necesitan menos células funcionales, ya que el objetivo es suplir un único enzima ausente. Las más habituales son las relacionadas con el ciclo de la urea, pero también hay varias pruebas clínicas que han demostrado la eficacia del trasplante de hepatocitos para otros casos como el síndrome de Crigler-Najjar, la deficiencia de almacenamiento de glicógeno o la hipercolesterolemia familiar, entre otros. Para otros casos en los que los pacientes sufrían de fallo hepático agudo o enfermedades crónicas los resultados no han sido tan positivos, demostrando que aún faltan muchos estudios para alcanzar el éxito en este tipo de terapias.

Otra de las alternativas que se están estudiando para el tratamiento de afecciones hepáticas es el uso de hígados bioartificiales. Son dispositivos extracorpóreos que pueden ayudar a los pacientes hasta que regeneren su propio hígado o hasta que reciban un trasplante. Esencialmente, son biorreactores que contienen células hepáticas que desarrollan las funciones de un hígado normal y procesan el plasma del paciente de una manera similar a soportes del tipo diálisis. Hay varios estudios con gran número de pacientes que demuestran la seguridad del procedimiento y su eficacia a la hora de mejorar las perspectivas de supervivencia de los pacientes. Sin embargo, el alto coste, complejidad, y dificultad a la hora de obtener células funcionales para el dispositivo han hecho difícil su aplicación en clínica. El punto más conflictivo reside en las células usadas para el procedimiento, ya que normalmente los hepatocitos, que serían los mejores candidatos, se usan para el trasplante directo. Las alternativas que se han usado más frecuentemente en estos biorreactores son hepatocitos de cerdo, ya que comparten muchas (aunque no todas) de las funciones de los hepatocitos humanos. Líneas tumorales hepáticas también se han aplicado con relativo éxito. Sin embargo, ninguno de los candidatos suple completamente todas las funciones de un hepatocito humano.

Debido a la gran capacidad de regeneración del hígado, es lógico pensar que en el propio hígado debe haber una fuente de células capaces de dar lugar a células hepáticas y que, si somos capaces de extraer estas células, podríamos solucionar muchos de los problemas relacionados con enfermedades hepáticas. Sin embargo, a diferencia de otros órganos que se pueden regenerar, el hígado normalmente no se regenera mediante una población de células progenitoras, sino que son los propios hepatocitos maduros los que se dividen y se encargan de regenerar el tejido que falte. Desafortunadamente, esta capacidad de división de los hepatocitos se ve superada cuando el daño hepático es muy grande o cuando hay enfermedades crónicas. En estos casos se activa una población de células específica, conocida como células hepáticas progenitoras. Estas células son bipotenciales y pueden dar lugar tanto a hepatocitos como a colangiocitos. Tienen un fenotipo conocido y se pueden aislar de pacientes de todas las edades haciendo una selección immunológica. Sin embargo, la aplicación clínica de estas células está limitada por la dificultad de obtenerlas en gran cantidad.

Células similares a las progenitoras hepáticas son los hepatoblastos, que se pueden obtener de hígados fetales. También son bipotenciales y podrían usarse en las mismas aplicaciones, pero las dificultades que presentan son las mismas: número bajo de células *in vivo* y difícil expansión el cultivo.

Por esta falta de éxito en la generación de hepatocitos a partir de células progenitoras hepáticas, se han buscado otras alternativas en células madre que *a priori* no tienen nada que ver con el hígado.

Las células madre se definen como células no especializadas que se caracterizan por su habilidad de dividirse indefinidamente y, a su vez, de generar otras células maduras con funciones especializadas. En humanos, las células madre se han identificado en innumerables zonas: en la masa celular interna de los blastocitos, en tejidos fetales como el cordón umbilical y la placenta y, también, en muchos órganos adultos. Los avances en la investigación con células madre han generado gran interés por la posibilidad de su aplicación en terapias para regenerar tejidos y órganos.

Las células madre embrionarias (ESC) son las que se obtienen de la masa celular interna de los blastocitos. Son células pluripotentes ya que pueden generar cualquier tejido de las tres capas embrionarias y tienen una expresión génica concreta que las caracteriza. No obstante, los problemas éticos derivados de su obtención así como su capacidad de formar tumores, las dejan lejos de su posible aplicación terapéutica.

Por otro lado, y como alternativa a las ESC en medicina regenerativa, están las células madre adultas, que se encuentran en diversos órganos del cuerpo humano, entre ellos: médula ósea, corazón, cerebro, pulmones, hígado, piel, ojos, etc.

Un grupo de células madre adultas, las células madre mesenquimales, ha generado un gran interés en el campo de la medicina regenerativa debido a sus propiedades immunomoduladoras. Fueron descubiertas en la médula ósea, pero actualmente se encuentran en varios tejidos como el tejido adiposo, el cordón umbilical o la pulpa dental.

Concretamente, en los dientes se han aislado varias poblaciones de células mesenquimales dependiendo de su ubicación concreta: las células madre de la pulpa dental (DPSC), células madre de dientes exfoliados (SHED), células madre del ligamento periodontal (PDLSC) y las células madre de la papila apical (SCAP). Todas estas poblaciones tienen características similares, pueden diferenciarse en osteoblastos, condroblastos y otros tipos celulares, aunque su capacidad de diferenciación es limitada. Se comparan normalmente con las células mesenquimales de la médula ósea ya que son las más conocidas.

Recientemente, nuestro grupo de investigación ha descrito el aislamiento de una nueva población de células madre adulta llamadas células madre pluripotentes de la pulpa dental (DPPSC). Estas células se obtienen de la pulpa dental de los terceros molares, expresan marcadores de pluripotencia tales como OCT4, NANOG y SOX2 y además son capaces de generar tejidos de las tres capas embrionarias, al igual que las ESC. Los terceros molares son una fuente accesible de células ya que se extraen frecuentemente y se consideran un desecho médico. Además, al ser el último órgano en desarrollarse, el tercer molar está en un estadio de

desarrollo inicial y se puede obtener un número elevado de células madre. Las DPPSC se diferencian de otras células de la pulpa dental en la expresión de los genes embrionarios así como la expresión de algunas proteínas de membrana como CD73. Las condiciones de cultivo de estas células son muy específicas ya que necesitan una baja densidad y un medio muy concreto.

Hasta el momento, han sido muchas las aproximaciones realizadas por diversos grupos de investigación para obtener hepatocitos a partir de células madre. Los primeros resultados fueron obtenidos a partir de experimentos con ESC ya que se conocía su gran plasticidad. Al principio, los protocolos de diferenciación pasaban por la formación inicial de cuerpos embrionarios (EB) para generar los hepatocitos, pero, más tarde, se descartó su uso debido a la heterogeneidad de las células obtenidas. Posteriormente, y basados en el conocimiento del desarrollo embrionario del hígado, se han generado protocolos en los cuales se induce la generación de hepatocitos mediante el uso de factores de crecimiento. Los protocolos normalmente usan Activina A, Wnt3a, proteínas de la familia de los factores de crecimiento de fibroblastos (FGF) y las proteínas morfogénicas del hueso (BMP) en los primeros estadios de la diferenciación. En pasos posteriores de la diferenciación, se utiliza el factor de crecimiento de hepatocitos (HGF), el FGF4 para inducir el linaje hepático y, finalmente, dexametasona (Dex) y Oncostatina M (OSM) para la maduración hepática. Sin embargo, las combinaciones de factores son prácticamente infinitas y los resultados pueden variar según el tiempo en el que se aplican y otros suplementos que pueda incluir el medio.

Con el reciente descubrimiento de las células madre pluripotentes inducidas (iPSC) se ha abierto otra alternativa al uso de las ESC. Los resultados en ambos casos son similares, ya que las células tienen características y potencialidad similar. A pesar de algunos resultados esperanzadores, las células obtenidas no se pueden considerar iguales a los hepatocitos maduros. Además, tanto las iPSC y la ESC presentan grandes riesgos a la hora de ser aplicadas en terapias debido a su capacidad para generar tumores.

Debido a estas dificultades, grandes esfuerzos se han realizado para generar hepatocitos a partir de células madre de origen adulto. A pesar de que la plasticidad de las mismas se creía muy limitada, se ha demostrado en los últimos años que son capaces de generar más tejidos de los esperados. En el caso de la generación de hepatocitos, sin embargo, los resultados no son tan satisfactorios como con las células embrionarias. Las células obtenidas no están tan bien caracterizadas y en muchos casos no cumplen con todas las funciones típicas de los hepatocitos.

Por eso, gracias a la gran capacidad demostrada por las DPPSC para generar tejidos de todas las capas embrionarias y a las ventajas que ofrecen por ser células adultas, creemos que pueden ser una fuente adecuada de células para la generación de hepatocitos aplicables en las distintas terapias para tratar afecciones del hígado.

Resultados

Con este trabajo, hemos demostrado algunas de las diferencias fenotípicas de las DPPSC y las células mesenquimales de la pulpa dental (DPMSC). Las DPPSC son más pequeñas y con una forma triangular mientras que las DPMSC son mayores y con una forma elongada. En cuanto a

la expresión génica, las mayores diferencias se encuentran en la expresión por parte de las DPPSC de genes embrionarios tales como OCT4, NANOG y SOX2.

Además, las DPPSC han demostrado su capacidad de generar diversos tejidos adultos cuando son inyectadas en ratones inmunodeficientes. Son capaces de derivar hacia tejidos de las tres capas embrionarias después de 5 semanas inyectadas subcutáneamente. Sin embargo, parece que todavía hay células indiferenciadas después de estas semanas, por lo que la derivación de tejidos no es completa.

Otra prueba que demuestra la gran potencialidad de las DPPSC es la formación de EB. Mediante varias aproximaciones, hemos conseguido generar estos EB a partir de DPPSC. A pesar de que las células siguen expresando genes embrionarios después de generar los EB, es importante remarcar que los genes característicos de las tres capas embrionarias aumentan sustancialmente, como sería de esperar en un EB.

Debido a que el método de selección de las DPPSC es relativamente permisivo, ya que solo consiste en cultivarlas en el medio específico que permite su crecimiento, quisimos ver si había otras células en cultivo al mismo tiempo que las DPPSC. Para ello, buscamos la expresión de CD73, un marcador típico de células mesenquimales, y vimos que había cierta parte de la población que expresaba esa proteína. Después de separar mediante citometría de flujo las dos poblaciones, vimos que las células que eran negativas para CD73 expresaban genes embrionarios como OCT4 y SOX2, mientras que las células positivas para CD73 no los expresaban. Esto demuestra que las dos poblaciones pueden convivir en cultivo pero que, a pesar de ello, son independientes.

Lo siguiente que quisimos averiguar fue si las condiciones de cultivo son determinantes en el fenotipo de las DPPSC y las DPMSC. Para ello, cambiamos las condiciones de cultivo de ambas poblaciones a las de la otra población y pudimos ver que la morfología de las células cambiaba sustancialmente, así como la expresión de genes embrionarios, de lo cual se deduce que las condiciones de cultivo afectan al fenotipo de las células.

Los siguientes experimentos se centraron en la obtención de células hepáticas a partir de las DPPSC. El primer paso para ello fue la combinación de diversos factores de crecimiento que se sabe que están involucrados en la generación de endodermo definitivo. Se estudió la expresión génica de las células después de cinco días expuestas a diversas combinaciones de factores y se pudo ver que había tres combinaciones que eran las más adecuadas para obtener endodermo: Activina A, Activina A combinada con Wnt3a y Activina A combinada con BMP4. Al ser todas igual de efectivas, a partir de aquí se optó por la utilización únicamente de la Activina A por razones económicas.

A continuación, se exploró el efecto de diversos elementos en la generación de endodermo definitivo con la Activina A. Estos factores fueron: la ausencia de suero fetal bovino (FBS), el uso de fibronectina como matriz de soporte alternativa y el co-cultivo con una línea celular de hepatoma (HepG2). Los resultados muestran que el FBS debe estar presente los tres primeros días de diferenciación para que las células generen endodermo eficientemente. El uso de fibronectina como matriz de soporte no supone diferencias significativas respecto al uso de colágeno I. El co-cultivo con HepG2 fue negativo para el desarrollo del endodermo, ya que las

células crecieron desmesuradamente y adquirieron una morfología diferente a la esperada, la cual se correspondió con la baja expresión de genes de endodermo.

Una vez la generación de endodermo estaba optimizada, los siguientes experimentos se basaron en el uso de un andamio de cristal para inducir la diferenciación de las células en hepatocitos maduros. Sin embargo, los resultados no fueron positivos ya que, aunque las células sí expresaban genes hepáticos durante los primeros estadios de diferenciación, luego eran incapaces de expresar los genes correspondientes a las fases más avanzadas. Así, la expresión de genes de hepatocitos maduros como CYP7A1 o HNF6 no era detectable después de tres semanas de diferenciación.

A partir de aquí se descartó el uso de la matriz de cristal y se optó por un entorno bidimensional para las células. Se comprobó la expresión génica de las células después de dos semanas de diferenciación y se vio que las células expresaban varios genes hepáticos tales como AAT, AFP, G6P, HNF6, Foxa1, entre otros, los cuales no eran detectados mediante RT-PCR cuando sólo habían pasado 7 días de diferenciación. Para intentar acelerar el proceso, decidimos comprobar el efecto que tenía en la expresión de los genes una exposición de Activina A durante 7 días en lugar de los 5 días del protocolo inicial. Vimos que, a diferencia de con la exposición corta, después de 7 días de exposición las células eran capaces de expresar tanto Foxa1 como AFP, lo que indicaba una acelerada entrada en el ciclo de diferenciación hepática.

Con esta mejora, el siguiente paso fue ver cuál era la expresión de las células diferenciadas después de 22 días de cultivo con su respectivo medio de diferenciación. Se estudió la evolución de varios genes y se vio que, al final del proceso, las células tenían un pico de expresión de genes típicos de hepatocitos maduros como AAT, ALB y G6P. Sin embargo, se apreciaba un gran número de células muertas al final del proceso de diferenciación. Para intentar disminuir este efecto, se utilizó un revestimiento con una combinación de proteínas extracelulares comercial (Geltrex). Los resultados demostraron que el uso del revestimiento con Geltrex era beneficioso para la supervivencia de las células, pero, también se observaba un efecto en la expresión de los genes hepáticos que no resulta tan positiva. Mediante qRT-PCR se pudo observar que la expresión de genes tales como AAT, ALB y G6P disminuía cuando era aplicado el revestimiento. Por este motivo, se tuvo que descartar el uso de esta técnica de cultivo, aunque puede que en el futuro tenga resultados positivos en el mantenimiento del cultivo de las células diferenciadas.

Tras descartar la utilización del revestimiento en la diferenciación, se quiso comprobar que, con el protocolo original, las células se habían diferenciado completamente. Por un lado, se comprobó la expresión de proteínas mediante técnicas de inmunofluorescencia e inmunocitoquímica. Los resultados indicaron que había expresión de proteínas hepáticas como ALB, AAT, AFP después de 22 de diferenciación.

Además, había que confirmar que las células diferenciadas, aparte de expresar los genes y proteínas hepáticas correspondientes, eran capaces de cumplir con las funciones esperadas de un hepatocito. Por ello, analizamos la secreción de ALB, la cual iba creciendo a lo largo de la diferenciación hasta que llegó a su máxima expresión después de 19 días de diferenciación. Por otro lado, mediante la tinción de ácido periódico de Schiff, se detectó que las células

adquirian la capacidad de almacenar glicógeno en su interior. Por último, las células diferenciadas expresaban CYP3A4, observándose que éste era funcional, ya que se pudo inducir su expresión mediante la estimulación con rifampicina.

Discusión

Varias poblaciones de células se han aislado a partir de los dientes humanos, aunque hasta el descubrimiento de las DPPSC, todas compartían las propiedades de células mesenquimales. Hemos demostrado que ambos tipos celulares pueden estar presentes en cultivo al mismo tiempo. Ya que las DPPSC no expresan CD73, proponemos el uso de esta proteína como método de selección para purificar cualquiera de las dos poblaciones.

Nuestros resultados demuestran, además, que las DPPSC se distinguen de las DPMSC por la expresión de genes embrionarios tales como OCT4, NANOG y SOX2. Estos genes se creían exclusivos de células embrionarias, pero en los últimos años se ha visto que hay varias poblaciones adultas que comparten estas características. La mayoría se encuentran en la médula ósea, como las células multipotentes adultas progenitoras (MAPC) o las células muy pequeñas parecidas a embrionarias (VSEL). En los dientes también se han encontrado poblaciones similares, pero siempre partiendo de dientes de leche.

Hemos demostrado que las DPPSC necesitan estar en las condiciones de cultivo adecuadas para mantener su fenotipo característico. Se ha visto que en otras poblaciones pasa algo similar, por ejemplo en células mesenquimales y MAPC. Faltaría por descubrir si estos cambios en las condiciones de cultivo también pueden afectar a la capacidad de diferenciación de las DPPSC.

La capacidad de formación de EB es un aspecto importante a la hora de demostrar la pluripotencia de las células. En nuestro caso hemos demostrado que las células son capaces de incrementar la expresión de genes específicos de las tres capas embrionarias después de estar en formación de EB. Sin embargo, parece ser que estas estructuras no están completamente desarrolladas, ya que mantienen la expresión de genes embrionarios. Este suceso se ha visto previamente en otras poblaciones, por ejemplo en ESC de ratón, donde se ha visto que las células recuperan la expresión de OCT4 y la mantienen durante más de dos meses a pesar de estar formando EB.

La relación entre las DPPSC y las iPSC también debería ser estudiada. Algunos estudios demuestran que células con elevada expresión de SOX2 o c-Myc tienen mayor éxito de reprogramación. Ya que las DPPSC tienen elevados niveles de genes embrionarios, es presumible que también tendrán más facilidad a la hora de reprogramarse o que requerirán menos factores para convertirse en iPSC.

En cuanto a la capacidad de diferenciación a células hepáticas, las DPPSC han demostrado ser una buena fuente de células madre para tal propósito. El primer paso para tal objetivo es la formación del endodermo definitivo. Hemos demostrado que la Activina A es un buen inductor de las DPPSC para tal fin y que no se requiere señalización de otras vías como pueden ser las inducidas por Wnt3a o BMP4. Además, hemos visto que los efectos de otras proteínas como FGF4 o bFGF en estos estadios iniciales no son positivos para la diferenciación. Hay otros factores que pueden estar involucrados en la obtención de endodermo definitivo que no han

sido explorados en este trabajo, como pueden ser BMP2 o FGF2. Sin embargo, la falta de estudios que demuestren su eficacia en otros tipos celulares hizo que fueran descartados para su uso en la diferenciación de las DPPSC.

La concentración de FBS parece tener un rol importante en estos primeros pasos de la diferenciación. Algunos estudios dicen que es necesario durante los primeros 5 días de la diferenciación, mientras que otros sostienen que con 3 días es suficiente. Nosotros hemos demostrado que, a partir del tercer día, puede ser sustituido por un equivalente de origen no animal pero que durante esos tres primeros días sí es necesario.

La especificación hepática es el siguiente de los pasos que deben seguir las células una vez convertidas al endodermo. En nuestro protocolo se usan el factor de crecimiento de hepatocitos (HGF) y el factor de crecimiento de fibroblastos 4 (FGF4) para tal función. Ambos se conocen como grandes partícipes en el desarrollo del hígado *in vivo*. Además, varias publicaciones previas han demostrado su importancia en la obtención de células *hepáticas in vitro*, por ejemplo aumentando la expresión de ALB. En nuestros experimentos se demuestra que los genes típicos de hepatocitos se empiezan a expresar después de 14 días, es decir, una vez ya han entrado en contacto con estos dos factores, lo que demuestra su influencia a la hora de inducir el fenotipo hepático.

El último paso es el de la maduración hepática, donde las células que ya tienen un destino hepático deben adquirir la capacidad de desarrollar las funciones hepáticas necesarias. Varios factores son reconocidos por su papel en este aspecto tanto *in vivo* como *in vitro*. La oncostatina M (OSM) es uno de ellos y es una citoquina que requiere de la acción conjunta de glucocorticoides como la Dex para desarrollar su función. En nuestros experimentos se usan ambos factores para inducir la maduración hepática y se demuestra que los mayores cambios morfológicos y la adquisición de funciones tales como la secreción de albúmina y la actividad GGT y AST se producen después de entrar en contacto con estos factores.

Son varios los estudios que demuestran que diferentes poblaciones de células madre se pueden diferenciar a células similares a hepatocitos. Sin embargo, los criterios a la hora de determinar si una célula es un hepatocito no han sido estandarizados y difieren mucho según el estudio. Esto hace que, a pesar de los resultados prometedores obtenidos hasta ahora con diferentes poblaciones de células madre, sean todavía insuficientes para su uso en aplicaciones clínicas.

No obstante, algunas pruebas son las más usadas a la hora de caracterizar una célula como hepática: evaluación de la morfología de las células, cuantificación de la expresión génica y proteica, testar la funcionalidad de las células y, finalmente, evaluar su comportamiento *in vivo*.

En nuestro caso hemos cumplido con todos los aspectos de caracterización de las células, excepto su comportamiento en animales. Los cambios en morfología son fácilmente detectables por microscopía óptica. Se puede observar claramente como las DPPSC cambian su morfología durante los primeros días para finalizar adquiriendo la forma poligonal típica de los hepatocitos y formando uniones entre las células, importantes para la polarización de las mismas.

La expresión génica también ha sido cuantificada y se ha podido observar como la expresión de los genes, tanto de endodermo como de hepatocitos, se corresponde con la esperada según la fase de la diferenciación. Por ejemplo, en los primeros estadios las células expresan Foxa1, Foxa2, AFP y Gata4 mientras que, más tarde, expresan ALB, AAT y G6P. Esta expresión génica se ha confirmado evaluando la expresión de las proteínas de algunos de estos genes, por ejemplo, se ha visto que las células son capaces de expresar proteínas de estadios iniciales como AFP, pero también de estadios más avanzados de la diferenciación, como AAT y G6P.

Finalmente, la evaluación de la funcionalidad de las células ha quedado clara con la capacidad de las mismas de secretar albúmina, su capacidad de almacenar glicógeno y de producir citocromo P3A4 activo e inducible. Este último aspecto es, probablemente, uno de los más importantes, ya que las células hepáticas se pueden usar para evaluar la toxicidad de medicamentos *in vitro* antes de pasar a hacer pruebas con ellos en animales. Por lo tanto, obtener unas células que tengan una buena actividad del citocromo P puede ser un paso importante para la sustitución del uso de hepatocitos primarios.

En conclusión, los resultados presentados en este trabajo demuestran que las DPPSC son una fuente prometedora para obtener hepatocitos funcionales, aunque para aplicaciones clínicas todavía quedan muchos estudios que realizar.

CEIC Comitè Ètic d'Investigació Clínica
Clínica Universitària d'Odontologia
Universitat Internacional de Catalunya

CARTA APROVACIÓ ESTUDI PEL CEIC

Número de l'estudi:B-27-EFP-09
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Títol: "Evaluación de la capacidad regenerativa de células madre de la pulpa dental"

Sant Cugat del Vallès, 27 de gener de 2011

Dr. Maher Al-Atari

Referència:

Benvolgut Doctor,

Els membres del CEIC de la Clínica Universitària d'Odontologia, li agraeixen l'aportació científica en el camp de la investigació i la presentació del Protocol en aquest Comité per a la seva avaluació.

Valorades les noves aportacions realitzades a l'estudi, sol.licitades pel nostre CEIC, el passat dia 17 de gener de 2011, li comuniquem que el dictamen final ha sigut FAVORABLE.

Quedem a la seva disposició per a qualsevol dubte o aclaració al respecte.

Atentament,

Sra. Immaculada Puga
Presidenta CEIC

Universitat Internacional de Catalunya

Barcelona, 13 de febrero de 2013

Sr. Carlos Gil Recio
Doctor Sant Ponç, 137, 5è, 1a
08030, Barcelona

Estimado Sr.

Por la presente, le comunico que la Comisión Académica del Doctorado en Ciencias de la Salud, en la su sesión del 8 de febrero de 2013, y una vez estudiada su solicitud ha acordado:

Se acuerda admitir al Sr. Carlos Gil Recio al Periodo de Investigación del Doctorado en Odontología.

Se acuerda aprobar el Proyecto de Tesis titulado "Obtención de una población de hepatositos a partir de células madre pluripotentes de la pulpa dental", y nombrar al Dr. Maher Al Atari como Director de la Tesis y al Dr. Miguel Barajas como Codirector.

Adicionalmente, se le informa que la normativa de la UIC establece que debe obtener una evaluación favorable del Comité de Ética en la Investigación, antes de la puesta en marcha de la investigación.

Aprovecho la oportunidad para saludarlo cordialmente,

Jaime Oliver Serrano
Secretario Comisión Académica
Doctorado en Ciencias de la Salud

Universitat Internacional de Catalunya
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